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Effect of Cortisone on Natural Immunity to *Schistosoma mansoni* in Mice.* (23377)

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Investigators who have used mice(1) as experimental hosts in *Schistosoma mansoni* infections find that the average number of worms recoverable from matured infections does not usually approach 100% of the number of cercariae to which the mice are exposed. A 50% yield of adult worms is considered good. Some of the factors known to influence the degree of success of infection with a particular strain of mouse include variations in infective potential of different batches of cercariae(2) and variations in manner and route of exposure of the host to the parasite. Natural immunity factors undoubtedly are involved as well, but as yet there appears to be little information as to what the natural immunity factors are and when and how they operate(3). Previous work with other helminth parasite-host combinations(4,5) has demonstrated that cortisone provides a powerful tool with which to study the immunity relationships. Accordingly it was regarded as worthwhile to test the effect of cortisone upon an experimental *S. mansoni* infection. Because cortisone is known to have a limiting effect upon the lymphoid-macrophage system it was suspected *a priori* that the natural immunity of the mouse host would be weakened; however, the results of 3 separate experiments outlined below indicate that natural immunity was not weakened and, on the contrary, provide evidence that it may have been enhanced somewhat.

Materials and methods. Local strain white mice were used in Experiment 1, brown mice in Experiments 2 and 3. They were fed Purina dog checkers and water *ad libitum*. *S. mansoni* cercariae for infecting the mice were obtained from laboratory-reared snails (*Australorbis glabratus* of Puerto Rican origin) infected in the laboratory with miracidia hatched from eggs isolated from human stools. Exposure of the mice was by tail immersion, for one hour, to cercariae recently emerged from 7-22 snails, with the mice restrained in plastic holders(6). The numbers of cercariae were standardized and delivered into rain water in the infection tubes, using a semi-automatic pipette. Cortisone was cortisone acetate Merck ("Cortone") diluted with 0.85% NaCl to a strength of 10 mg per ml, given by the subcutaneous route in 0.5 mg

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TABLE I. Average Numbers of Worms Recovered from Treated and Control Mice, and Results of T-Tests of Mean Differences. NS = not significant.

Exp. No.	Avg No. worms recovered Cortisone	 Control	S.E. of mean diff.	Signif. level	Degrees of freedom
1	10.1	17.1	2.414	.05	12
2 A (♂)	19.7	28.5	4.760	NS	6
2 B (♀)	1.5	5.5	2.512	NS	6
3	16.9	23.9	2.067	.01	8 or 18

doses. Seven or 8 weeks after exposure to infection each mouse was killed by cervical fracture, the viscera carefully removed and the worms teased out in saline for counting under a low power binocular dissecting microscope, recording their occurrence in mesenteric vessels, main portal vein and liver. Spleen, kidneys and lungs were examined occasionally but not routinely; in the cases where they were examined no worms were found in them.

Experimental procedures. In Experiment 1, 14 mice (8 males, 6 females), all 8 weeks old, were divided into 2 groups of 7 mice each (4M, 3F per group) and exposed simultaneously to approximately 40 cercariae per mouse. Mice of the experimental group were given a total of 12 injections of cortisone on the following days with reference to infection: -2, 0, 1, 2, 6, 10, 13, 17, 20, 23, 26 and 29. In Experiment 2, 17 mice 7 weeks old were divided into 2 groups of 9 and 8 mice respectively (cortisone: 5M, 4F; control: 4M, 4F) and exposed to approximately 55 cercariae per mouse. Inadvertently all males were exposed first and the females an hour later. The cortisone group mice were given a total of 9 injections on days: -1, 0, 1, 3, 5, 7, 9, 11 and 15. In Experiment 3, 20 mice 15 weeks old were divided into 2 groups of 11 and 9 mice respectively (cortisone: 7M, 4F; control: 6M, 3F) and exposed simultaneously to 50 cercariae each. The cortisone group mice received a total of 7 injections on days: -1, 0, 1, 3, 4, 5 and 7.

Results. All mice survived to necropsy except for 1 male cortisone-treated mouse in Experiment 2, which succumbed on the 20th day after infection. The cortisone-treated mice lost weight during cortisone treatment and appeared somewhat debilitated where the treatments were of extensive duration as in Experiment 1, but after treatments ceased they soon regained normal appearance and vigor comparable with the controls. A summary of average total worm counts in each experiment is given in Table I along with results of statistical tests of differences between the means. In all 3 experiments fewer worms were recovered from the cortisone-treated mice than from the controls. The differences between the means of treated and control groups were statistically significant in Experiments 1 and 3. In Experiment 2 far fewer worms were recovered from female mice of both groups than from the male mice. This does not represent a true sex difference, in view of the results of Experiment 3 and of another experiment not reported here, both of which utilized the same strain of brown mice. As previously indicated, the female mice were inadvertently exposed to infection at a later time than the males, although on the same day from the same pool of cercariae, and the cercariae apparently lost much of their infectivity even though this is not normally the case where the time between exposures is so short.

Discussion. These 3 experiments were closely but not exactly similar in design. In all of them cortisone treatments began prior to infection but the treatments were continued for progressively shorter periods of time, terminating on days 29, 15 and 7 respectively. The consistent trend of the results shows definitely that the regimens of cortisone treatment employed did not lessen the natural resistances of the treated mice to *S. mansoni* as measured by numbers of worms recovered from the hepatic portal tracts of the mice 7-8 weeks after infection. There is, moreover, evidence strongly suggestive of an opposite effect, since in all 3 cases fewer average numbers of worms were recovered from the cortisone-treated mice than from the untreated control mice. It is possible, but not likely, that worms may have ended up in other portions of the vascular system where they could have been overlooked; they did not accumulate in spleen, lungs or kidneys. This apparent strengthening of immunity stands in sharp contrast to findings with 2 nematode parasites, *Trichinella spiralis*(5,7) and *Nippostrongylus muris*(4), where cortisone treatment of the host weakens both natural and acquired resistances presumably because the treatment restrains the cellular phase of the immunity mechanism(8). By inference it is likely that in mice cellular defenses play little part in the initial phases of natural immunity to *S. mansoni,* that is during the period of the transient residence of the larva in the skin tissues. If the immunity strengthening effect observed here is a true one, then from the results of Experiment 3 where treatment ended on the 7th day after exposure to the parasite it would appear that the effect is exerted in the very early stage of infection and may be related to an inhibition of skin penetration and/or further migration by the larvae. Cercariae are known to exhibit hyaluronidase activity(9), and cortisone is known to inhibit hyaluronidase(10). According to Lewert and Lee the infective larvae of both *S. mansoni* and *Strongyloides ratti* possess collagenase activity, and cortisone treatment of the rat host seemed to inhibit this activity of *S. ratti* and to cause longer retention of the young worms in the skin(11). These facts may not necessarily be related to the present findings, but certainly further investigation is in order on the effects of cortisone in schistosomiasis.

Summary. In 3 separate experiments small groups of mice of 2 strains were exposed to infection with *Schistosoma mansoni* and half of them were treated with cortisone beginning shortly prior to infection and ending on the 29th, 15th and 7th days after infection, respectively. The various regimens of cortisone treatment employed did not increase susceptibility to the parasite, as judged by numbers of adult worms recovered from hepatic portal tracts of the mice 7-8 weeks after infection. The treatments may have enhanced the natural immunity slightly, since significantly fewer worms were recovered from the cortisone-treated mice.

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Paper Electrophoresis of Muscle Fractions from Vitamin E Deficient Rabbits.* (23378)

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These studies were undertaken in an attempt to correlate vit. E avitaminosis with changes in muscle proteins and excretion of urinary nitrogen components, such as creatine, allantoin and amino acids. The easily soluble

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TABLE I. Average Daily Food Consumption and Weight Gain Data.

	Vit. E deficient (g)	Vit. E (g)
Food consumption	26.9	31.5
Wt gain	7.5	15.8

muscle proteins were studied by means of paper electrophoresis. This method was found to be sufficiently sensitive to differentiate muscle from dystrophic and control animals.

Experimental. Three pairs of male littermate California Giant rabbits weighing about 800 g were divided into 2 groups, one of which was fed a vit. E deficient diet and the other a vit. E supplemented diet. To this commercial diet† was added 2% codliver oil. Three milligrams of dl-α-tocopherol phosphate disodium salt per 25 g of the diet was fed on alternate days to the control group. The vit. E deficient group received the unsupplemented diet. The average daily food consumption and weight gain appears in Table I.

When the vit. E deficient animal of each pair showed typical signs of vit. E deficiency (1) the pair of animals was sacrificed by bleeding under anesthesia (ether). The eviscerated carcass was stored in the cold room and within 10 days striated muscle was removed from the right rear leg and ground in a small hand meat grinder. Twenty-five gram portions of ground muscle were extracted with 25 ml of distilled water, for 2 hours, at room temperature with occasional stirring. The mixture was then filtered through triple thickness gauze, centrifuged for 30 minutes at 450 x g. The supernatant fluid was recovered for the electrophoretic examination.

Filter paper electrophoresis was carried out using a glass plate horizontal electrophoresis apparatus and Whatman No. 3 filter paper with a barbiturate buffer pH 8.6 containing sodium diethyl barbiturate (20.6 g/l) and diethylbarbituric acid (3.68 g/l). Ten strips were run simultaneously using 20 lambda of the solution spotted at the midpoint of the strip. A potential of 450 volts with a current of 12 to 15 ma was applied for 3 hours, giving a separation of 5 to 6 cm between the more rapidly moving and the more slowly moving components. The strips were oven dried at 110°C for 30 minutes, then stained with a 1% solution of bromphenol blue in 95% ethyl alcohol saturated with mercuric chloride. The strips were placed in the dye bath for 5 minutes and washed free of excess dye by four 5 minute washings with methyl alcohol containing 1% mercuric chloride, with frequent agitation of the container. Finally, the strips were washed with methyl alcohol to remove the mercuric chloride and air dried on a clean dry cloth.

A line was drawn through the center of the strip lengthwise and was ruled off every ⅛ of an inch and optical density was determined at each intersection of the lines in a Photovolt Densitometer 52c without a filter. The results of such an analysis appear in Table II. These data show a significant difference in distribution of protein between the dystrophic and the control groups in the fast moving component and a significant difference in the slow moving component.

The electrophoresis pattern of the water extracts of a similarly treated pair of animals was stained and cut each ⅛″, and was eluted with 3 ml of 4% sodium carbonate in 50% methyl alcohol for 2 hours. The optical density of the eluate was read at 540 mμ and optical density x 1000 was plotted on the ordinate and the fraction number on the abscissa in Fig. 1.

Discussion. The findings reported here, using a water extraction procedure, showed that vit. E deficiency caused a change in the electrophoretic pattern of the easily soluble muscle proteins. Amount of nitrogen extracted from the muscles was the same in the control and the dystrophic group. Haan(2),

TABLE II. Optical Density of Water Extract of Muscle Electrophoretic Pattern.

	Vit. E deficient	Vit. E
Total optical density	5.14	5.17
% of total		
Fast moving	31.1	23.3
Slow "	68.8	76.6
2P (comparison of slow moving)=	.025	
2P (" " fast ")=	.02	

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† Nutritional Biochemicals, Cleveland, Ohio.

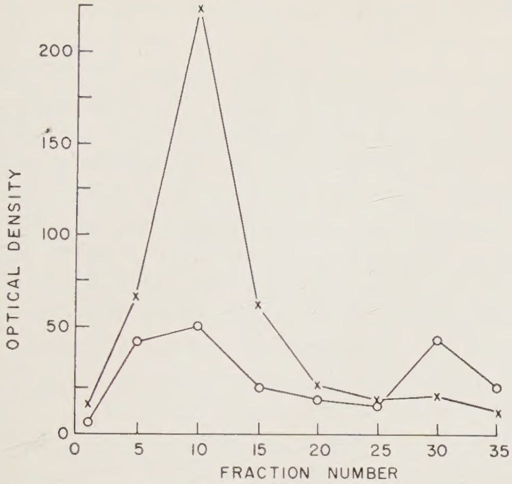

FIG. 1. Electrophoresis of aqueous extract of muscle from vit. E supplemented (×) and vit. E deficient (○) animal.

employing a dilute salt extraction procedure found that there was an increase in percentage of the most rapidly moving component peak 11 and also that the amount of the fast moving component peak 11 was constant in both groups of animals.

Summary. Three pairs of male littermate rabbits were fed a commercial vit. E deficient and a vit. E supplemented diet. When the vit. E deficient member of each pair showed typical vit. E deficient symptoms, the pair of animals was sacrificed and the water soluble muscle proteins extracted from the striated muscle. The electrophoretic pattern of the deficient animals showed a significant change in distribution of water soluble proteins as compared to the control group. This change was an increase in the fast moving peak and a decrease in the slow moving peak.

The authors wish to thank Mrs. B. A. Sullivan for assistance in the care of the animals.

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Ethanol Metabolism in Vitamin Deficiencies. I. Niacin.* (23379)

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Westerfeld(1) recently reviewed the pathways of ethanol† metabolism, and more recently Schulman and Westerfeld(2) have proposed an additional possible pathway. Since the route of metabolism has not been definitely established, it seemed worthwhile to study the effects of various vitamin deficiencies on the rate of alcohol metabolism in an effort to evaluate the role of vitamins. The first step in alcohol metabolism appears to be its conversion to acetaldehyde probably by means of alcohol dehydrogenase with DPN acting as hydrogen acceptor. Since DPN is a niacin-containing coenzyme, this study was begun with niacin.

Methods. Weanling male rats of the Sprague-Dawley strain, 24 days old when received in this laboratory, were used throughout the experiments.

Since the rat can synthesize niacin from abundant dietary tryptophan, the tryptophan content of the diet was limited. Preliminary work in this field indicated that a true niacin deficiency could be induced in rats with the diet shown on the next page.

The control diet contained, in addition, 50 mg niacin. Some rats were maintained on a diet similar to the above except the casein-casein hydrolysate ratio was 80-120 instead of 100-100.

Immediately upon arrival of the rats in this laboratory, the animals were paired and individually caged, and the deficient group was allowed to eat the niacin deficient diet

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† Hereafter referred to as alcohol.

Casein	100	g
" hydrolysate (tryptophan free)	100	g
Sucrose	700	g
Cottonseed oil	40	g
Cod liver oil	10	g
Alphacel	20	g
Salt mixture (U.S.P. XIV)	30	g
Thiamine hydrochloride	5	mg
Riboflavin	5	mg
Calcium pantothenate	10	mg
Pyridoxine hydrochloride	10	mg
Para-amino benzoic acid	50	mg
i-inositol	250	mg
Choline chloride	1500	mg
Biotin	.2	mg
Pteroylglutamic acid	.2	mg

ad lib. At the end of each 24 hour period the food was weighed and amount of food eaten by a deficient animal was fed to his pair-fed control. A third and fourth group of animals were allowed to eat the 100-100 and 80-120 casein-casein hydrolysate control diets *ad lib.* These groups of animals were used for weight comparisons with the deficient animals. Rates of alcohol metabolism were determined only on deficient and pair-fed control animals.

When an animal was considered niacin deficient by comparison of his growth curve with that of the control animals fed *ad lib.*, alcohol metabolism was determined in the following manner: alcohol was injected intraperitoneally as a 20% W/V solution in isotonic saline in a dose of 3 g/kg. The injection was made with a calibrated syringe pipette (Vernes Rheometer). After injection the animal was allowed to metabolize for exactly 3 hours. He was then killed by a sharp blow on the back of the neck and cut into small pieces into an all-metal Waring blendor. Blood and any other remains of the animal were washed from the scissors into the container with a weight of water 7 times that of the animal. The container was sealed with its air tight metal screw cap and the 1 to 8 homogenate was made. After homogenization, the container was cooled in an ice bath before removing the cap. Alcohol concentration of the homogenate was determined by the method of Aull *et al.*(3), using the specially designed Aull alcohol apparatus.‡ Rate of alcohol metabolism was calculated by subtracting recovery of alcohol in mg/100 g rat after metabolism from 304, multiplying by 10, and dividing by 3. The rate is then expressed as mg alcohol metabolized/kg/hr. Recovery of alcohol from 12 dead rats weighing 50-68 g and injected as above was 304 ± 3.9 (mean ± standard deviation) mg/100 g rat. This value was used in the calculation of rate of metabolism as the initial dose instead of the theoretical value of 300 mg/100 g rat. Fed deficient animals had access to food until just before the injection of alcohol. Fed pair-fed animals were fed a few hours prior to the run. All fasted animals were denied access to food for 24 hours.

Results. The results of 19 pairs of animals are shown in Table I. In each of Groups I and II three pairs of animals were fed the diets containing the 80-120 ratio of casein-casein hydrolysate, while all other pairs were fed diets containing the 100-100 ratio. No difference was noticed between the 2 different ratios. As can be seen, some averages show a rather high standard deviation. This is in part due to the differences in age and weight within a group of animals; the younger, smaller animals tend to show a higher rate of metabolism. Nevertheless, in 18 of the 19 pairs of animals, the deficient animal exhibited a higher rate of metabolism than did his pair-fed control. The single deficient animal that showed a lower rate of metabolism is included in Group I. One might postulate that the deficient animals exhibit a higher rate of metabolism because they are smaller. However, if one calculates rate of metabolism as mg alcohol oxidized/whole animal/hr, one finds that in 16 of the 19 pairs the deficient animal still exhibits a higher rate of metabolism. It would be customary to expect a smaller animal to burn less alcohol per whole animal. Whether the animals were fed or fasted appears to have no significant effect on the over-all picture. Recent work by Smith *et al.*(4) indicated that injection of nicotinamide into mice 6 hours prior to injection of alcohol had no effect on rate of alcohol metabolism, although DPN concentration in the liver was raised 10-fold.

Summary. It has been shown that niacin deficiency in rats has the effect of increasing

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‡ Scientific Glass Apparatus Co., Bloomfield, N. J.

TABLE I. Effect of Niacin Deficiency on Rate of Alcohol Metabolism.

Group	No. of rats	Deficient or pair fed	Wt in g (range, avg)	Age in days (range, avg)	Fed or fasted	Rate of metabolism (mg/kg/hr), avg ± S.D.	p values
I	6	Def.	40-65 49.2	44-54 49.3	Fed	493 ± 39	<.10 >.05
	6	P.F.	46-68 54.0	44-54 49.3	”	401 ±106	
II	6	Def.	38-70 50.3	45-55 49.7	”	461 ± 25	<.01
	6	P.F.	46-66 52.2	45-55 49.7	Fasted	372 ± 63	
III	7	Def.	46-53 48.1	53-64 59.7	”	370 ± 41	<.01
	7	P.F.	46-53 50.9	53-64 59.7	”	313 ± 21	
I, II, III	19	Def.	38-70 49.1	44-64 53.3	—	438 ± 64	<.01
	19	P.F.	46-68 52.3	44-64 53.3	—	359 ± 76	
I, III	13	Def.	40-65 48.6	44-64 54.9	—	427 ± 74	<.05 >.01
	13	P.F.	46-68 52.3	44-64 54.9	—	353 ± 84	

rate of alcohol metabolism in spite of the fact that DPN is generally conceded to be involved in conversion of alcohol to acetaldehyde.

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Preparation of Coated Radioautographs by Dipping Sections in Fluid Emulsion.* (23380)

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In 1946, the coating method of radioautography was developed in collaboration with Bélanger(1). The intimate contact provided by this method between the photographic emulsion and the radioactive tissue section insured a high resolution, which has not been improved by any technical procedures, except by the use of high contrast "nuclear" emulsions. In 1947 it was proposed to mount the section directly on a photographic plate(2) or to place a stripping film over the section(3). A high resolution could also be obtained with these procedures(4), but the coating method(1,5) has been preferred by the present authors because of the speed with which large batches of slides may be handled. More recently, Joftes and Warren proposed a method in which the slide is dipped in melted emulsion(6). While several steps of the technic were felt to be cumbersome or unnecessary, the dipping itself is simple and requires a minimum of equipment. An attempt was, therefore, made to introduce a "dipping" step into the coating method routinely used here. The results proved to be highly satisfactory and the technic will now be described in detail.

Slides bearing histological sections, either stained or unstained, are placed in a 1% solution of celloidin for a few seconds ("Tissue Imbedding Solution" No. M 4700, manufactured by Randolph Products Co., N. J., di-

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This work was presented by the senior author before the Histochem. Soc. in Baltimore, April 15, 1957.

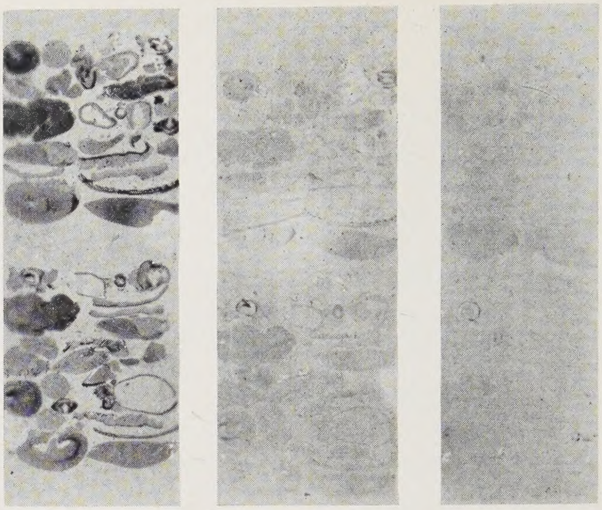
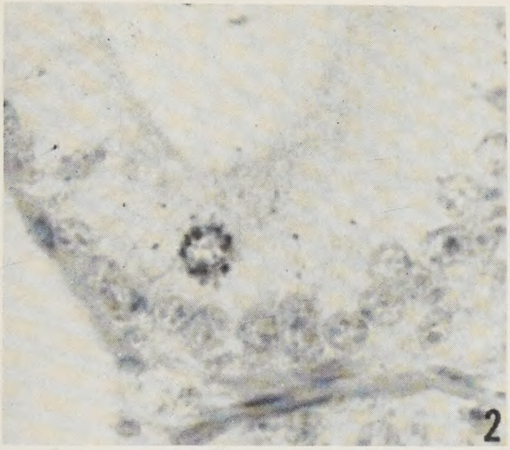


FIG. 1. Unstained radioautographs of tissues from mouse sacrificed 1 hr after inj. of S^{35}-methionine. Exposure at various humidity levels. After exposure in a dry atmosphere (left), reactions were intense, while at 40% humidity (center) reactions were weak and at 95% humidity (right) reactions were lacking. (Slightly darkened areas visible on right hand side are not due to reactions but to traces of picric acid retained in the Bouin fixed tissues.)

luted 30 times with a 1:1 mixture of ether and absolute alcohol). After drying for 10-15 minutes, the slides are placed in celloidin again for a few seconds. The celloidin coats preserve the staining to some extent during development and fixation, and also prevent the direct contact of the photographic emulsion with the tissue, thus minimizing chemical fogging by non-radioactive tissue components or dyes(4). After drying overnight at room temperature, the celloidin coated slide is smeared with a small drop of a 1:1 egg albumin:glycerine solution on either side of the sections, a procedure by which the emulsion is kept in place during development and subsequent handling.

The steps to follow are carried out at about 3 feet from a Wratten safelight No. 2 in a completely light-proof dark room. The temperature of the room is maintained at 60°F (16°C) and the humidity kept between 10-20% by leaving an open dish containing Drierite (a drying agent) in the room. The flask containing the stock solution of gelled emulsion (so far, bulk Eastman Kodak NTB 2 and NTB 3 have been used) is removed from a refrigerator kept in the dark room. A volume of emulsion estimated at about 60 ml is scooped out with a porcelain spoon and dropped in a Borrel tube standing in a water bath at 40-45°C. The emulsion is left in the water bath for at least 1 hour, by which time it is fluid and reasonably free of air bubbles (which otherwise produce artefacts in the emulsion coat). It was found useful, although not essential, to warm the slides on a hot plate at 40°C to allow a more uniform spreading of the emulsion. Then, the slides held at the label end are dipped for 1-2 seconds in the melted emulsion.

Attempts to use emulsion at 33°C failed as the emulsion coat was thick and unevenly distributed, while at 42°C and 60°C the layer had the desired thinness and uniformity. However, since temperatures above 50°C were found to induce bubble formation and increase the danger of fog, a temperature of 40-45°C was finally selected. After withdrawal of the slide from the fluid emulsion, the excess is allowed to drain into the container and the back of the slide is wiped clean with soft paper. The slide is then allowed to dry in a vertical position. It has been found that the emulsion layer hardened more uniformly when the slides were kept vertical than when laid flat. Drying is complete when the slide has been left standing for about 1½ hours (with the safelight turned off).



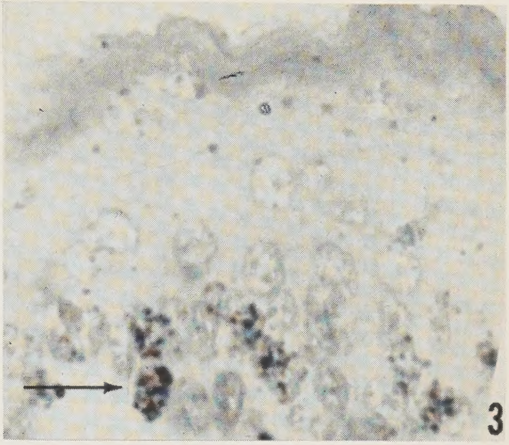


FIG. 2. Radioautograph of epididymis from mouse sacrificed 8 hr after inj. of H^3-thymidine. Hematoxylin and eosin stain. The nucleus of an epithelial cell in prophase shows a positive reaction. (In this, as in many epithelia, the nuclei move towards the lumen soon after prophase starts.) × 800.

FIG. 3. Radioautograph of tongue epithelium from mouse sacrificed 24 hr after inj. of H^3-thymidine. Hematoxylin and eosin stain. A positive reaction is visible over several of the nuclei in the basal layer. × 800.

The thickness of the emulsion was estimated to be 4 μ under the above conditions, but could be increased by dipping the slides 2, 3 or more times.

In seeking optimum condition for exposure, the influence of humidity on the radioautographic image was investigated as follows. A number of unstained sections from mice sacrificed 1 hour after injection of S^{35}-methionine were coated by dipping in NTB 2 emulsion as described above. The slides were distributed in 3 light-proof boxes kept at 5°C which, in addition, contained respectively: 1) Drierite to reduce the humidity close to 0%; 2) a beaker filled with a saturated solution of $CaCl_2 \cdot 6H_2O$ to yield an atmosphere with 40% humidity; and 3) a beaker filled with a saturated solution of $ZnSO_4 \cdot H_2O$ to obtain 95% humidity. After 7 weeks, the slides were developed and fixed simultaneously. It may be seen (Fig. 1) that the radioautographic reaction was intense in a dry atmosphere, weak at 40% humidity and absent at 95% humidity. Similar results were obtained with thyroid sections containing I^{131} and exposed for only 5 days, so that the inhibitory effect of humidity is just as pronounced after a short exposure. Since in both experiments the response was maximal in the absence of humidity, it was decided to expose all further radioautographs in a dry atmosphere. Routinely, the slides are stored in black plastic boxes, containing a perforated stainless steel capsule filled with Drierite. The box is sealed with black adhesive tape and kept standing on edge in the refrigerator (5°C) so that the slides are exposed in the horizontal position, emulsion side down.

After exposure, the preparations are developed for 1½ minutes in Eastman Kodak Dektol developer (D-72 type). Fixation is carried out for 10 minutes in acid fixer with hardener. The slides are then washed in running water at 18°C for 15 minutes and dehydrated in 95% alcohol and 2 changes of absolute alcohol.

For mounting radioautographs in permanent form, the slides are taken through absolute alcohol, rinsed in acetone for 5 minutes, placed for 5 minutes in a 1:4 acetone dilution of "vinylite cement" (General Cement Mfg. Co., Rockford, Ill.), and finally allowed to dry overnight at 60°C in the vertical position. The next day, the radioautographs are mounted by adding a drop of Canada balsam and covering the preparation with a coverslip (Fig. 2 and 3).

Summary. A simplified coating method for radioautography has been described in which the radioactive slides are dipped in melted

photographic emulsion. After exposure, the developed emulsion is protected with a coat of vinylite and mounted under coverslip.

Note added in proof: a dipping method of radioautography was also found in the German literature: Kolousek *et al., Acta Histochemica,* 1956, v3, 328.

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Utilization of Methylglyoxal in Animal Tissues. (23381)

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In spite of the difficulty of detecting methylglyoxal (MG) in the presence of the highly active enzyme glyoxalase, Neuberg and Kobel(1) isolated the compound from products of fermentation of hexose diphosphate (HDP) by dried yeast. MG is also formed from HDP by mixed preparations of muscle and pancreas (Tonniessen and Fisher(2), Ariyama(3)), and by acetone powders of rat liver (Artom(4)). Geiger and Rosenberg(5) detected MG in urine of polyneuritic dogs and in urine and cerebrospinal fluid of infants suffering from acute toxic dyspepsia; and Salem(6) could demonstrate MG in urine of thiamine-deficient rats. It appears therefore that MG is produced from HDP and can be detected under conditions, in which activity of the glyoxalase system is reduced or suppressed.

The aim of the present paper is to examine the possibility that MG could be converted enzymatically to HDP, *i.e.* to investigate whether the reaction: HDP→MG + Inorganic phosphate (Pi) can be considered as a reversible reaction.

Methods. MG was prepared from acetone by slight modification of the method of Riley, Morley and Friend(7). HDP was supplied by L. Light, Ltd., as the Ca salt. Fresh rat liver was weighed, ground with sand in a mortar, then extracted with one part of water and with occasional stirring for 30 minutes in the refrigerator. The mixture was centrifuged, and the supernatant used as a source of the enzyme. Pi was determined according to Fiske and Subbarow(8) and MG according to Salem(6). Two methods (a) and (b) were used to identify the compound resulting from incubation of liver preparation with MG: (a) Ba soluble and Ba insoluble phosphorylated compounds were fractionated, as described by Umbreit *et al.*(9), and hydrolysis curves were determined according to Lohmann(10); (b) descending one-dimensional paper chromatograms were made with trichloroacetic acid:acetone (35/65 v/v) as solvent. Molybdic acid method for phosphate esters (Hanes and Isherwood(11), Axelrod (12)) was used to detect HDP. Apparatus and working details were as described by Dent(13).

Results. Changes of Pi and MG during incubation: 1 ml of liver extract was incubated with 0.5 ml of 1:5 kidney extract for 2 hours at room temperature, to inactivate the glyoxalase system. Then 2 ml of 1% neutralized MG solution, 2 ml of borate-boric acid buffer, pH 7.0, and 0.2 ml toluene, as an antiseptic, were added. A similar sample was prepared, except that MG was omitted. Both samples were incubated at 37°C for 24 hours. The protein was precipitated with 10% trichloroacetic acid, and the amounts of Pi were determined on supernatants before and after incubation (with or without MG). From Table I, it is apparent that when MG was incubated

TABLE I. Inorganic P before and after Incubation, in 4 Experiments.

Before incubation	After incubation with methylglyoxal	After incubation without methylglyoxal
mg		
.23	.12	.23
.22	.11	.22
.20	.11	.21
.16	.11	.18

with a mixture of liver and kidney extracts, there was a decrease of about 50% in the Pi initially present. In some experiments, in which the MG remaining after incubation was also measured, it was found that nearly one-fourth of the original amounts had disappeared. It should be noted that glyoxalase was completely inactive, as shown by determining enzyme activity in mixed liver and kidney extracts. The amounts of MG disappearing were about 25 times greater than the equivalent amounts of Pi disappearing during incubation. This lack of equivalence is possibly due to interference of other reactions, such as liberation of Pi from other phosphorylated compounds, or destruction of MG through other pathways. At any rate, the simultaneous disappearance of Pi and MG is in line with the hypothesis of a reaction in which both compounds are used.

Fractionation and hydrolysis of incubation products. By the method we have used(9), phosphorylated intermediates are separated into a Ba insoluble fraction, which contains ATP, ADP, HDP and phosphoglyceric acid, and a Ba soluble fraction, containing glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, phosphopyruvate and triose phosphates. Hydrolysis curves of the 2 fractions allow the probable demonstration of the compound(s) present. In our experiments, 5 ml of the mixture of liver and kidney extracts were incubated at 37° for 24 hr with 10 ml of borate-boric acid buffer, pH 7.0, 10 ml of 1% MG, and 1 ml toluene. The controls "before" and "after incubation" were prepared identically, except that 10 ml of distilled water were substituted for the solution of MG. In the experimental and control samples, the Ba soluble and Ba insoluble fractions were separated and hydrolyzed, and Pi was determined on aliquots taken at various time intervals. Fig. 1 records the hy-

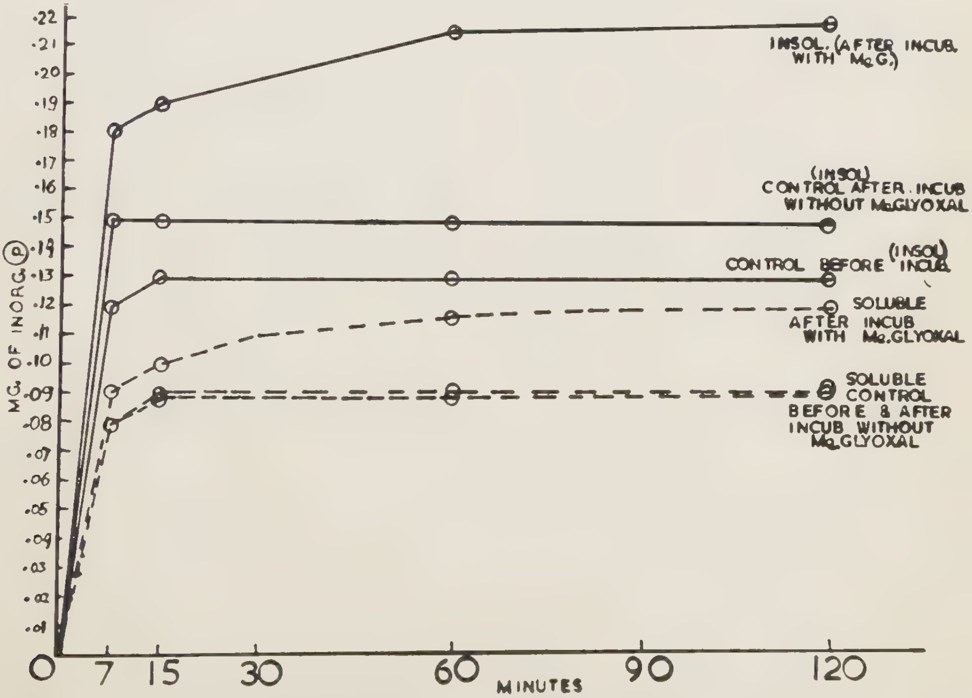

FIG. 1. Hydrolysis curves of barium soluble and insoluble fractions, before and after incubation with and without methylglyoxal.

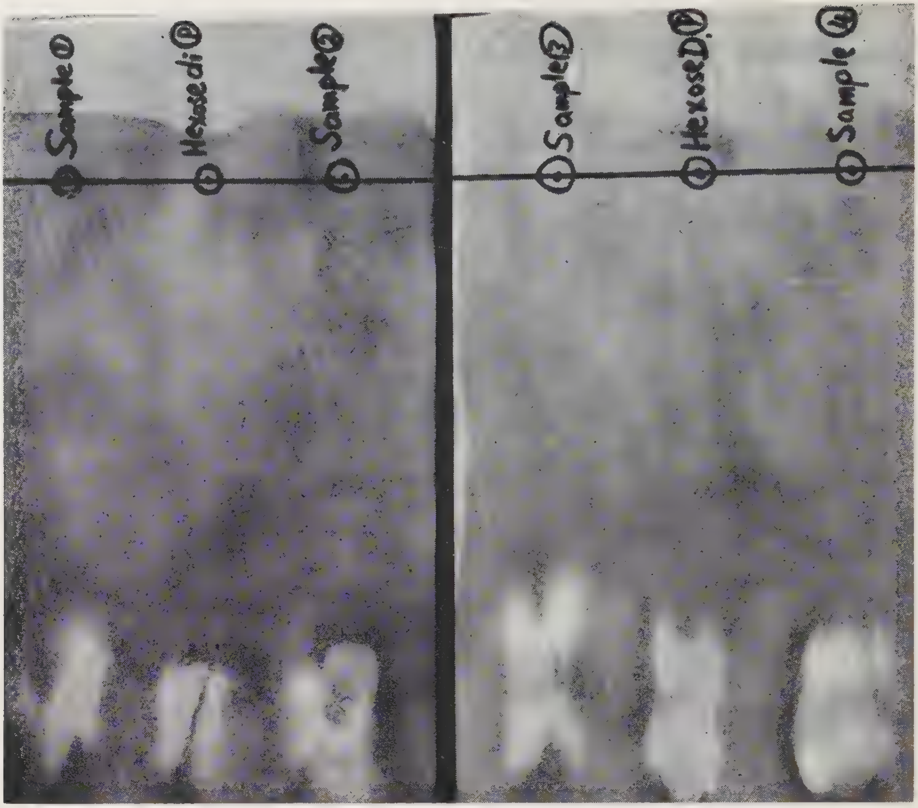

FIG. 2. Paper chromatograms of products of incubation of methylglyoxal with liver extracts (samples 1, 2, 3 and 4) and of solutions of pure hexosediphosphate (Hexosedi P). (No spots were detectable in the "controls" not incubated, or incubated without methylglyoxal.)

drolysis curves of the 2 fractions, as determined in one of our experiments. It is apparent that after incubation with MG, the increase in total hydrolyzable P of the Ba insoluble fraction was greater than that of the same fraction, incubated without MG. There was also an increase in total hydrolyzable phosphate of the soluble fraction after incubation with methylglyoxal, whereas there was no appreciable increase in the P of this fraction after incubation without MG. According to Umbreit *et al.*(9), 26.5% of HDP is hydrolyzed in the first 7 minutes, and then hydrolysis continues at a slower rate, whereas 46% of phosphopyruvic acid and triosephosphates is hydrolyzed in 7 minutes. The hydrolysis curve of the Ba insoluble fraction incubated with MG suggests therefore that HDP, or a P-containing compound with similar properties, increases during incubation with MG. The increase in the Ba soluble fraction curve might be due to formation of phosphopyruvic acid, or of triosephosphates, or both. It is possible that MG is converted to HDP through some steps including formation of triosephosphates. The slight increase in total hydrolyzable P of the Ba insoluble fraction after incubation without MG was unexpected. It may be noted that such increase occurred only during the first 7 minutes of hydrolysis.

Paper chromatography of incubation products. "Experimental" samples containing MG, liver and kidney extracts, borate buffer, and toluene were incubated at 37° for 24 hours. In addition, 2 "control" samples containing the extracts, the buffer and the antiseptic, but no MG were prepared. To one of these samples, trichloroacetic acid was added immediately, whereas the other control was incubated at 37° for 24 hours. After deproteinization and neutralization, small aliquots of the supernatants from the experimental and the control samples were placed on paper. At the same time, aliquots of the commercial solution of HDP were also chromatographed. With the molybdic acid reagent, one blue spot on bleached background was detected in the chromatograms of each of the experimental samples, the position of the spot being identical to that observed in the chromatograms of the HDP solution. No spots were detectable in the chromatograms of the control samples, nonincubated, or incubated with MG. The chromatograms of 4 experimental samples (numbered 1 to 4) and of 2 samples of the HDP solution (indicated as hexosedi-P) are shown in Fig. 2.

Discussion. The present results give at least presumptive evidence for a conversion of MG into HDP. This conversion would be in line with the observation of Stoehr(14) that liver glycogen is formed from MG, fed at a carefully controlled level. Moreover, if D-lactate is formed from MG by glyoxalase and if the action of this enzyme is also reversible, as claimed by Dakin and Dudley(15), the following scheme may be tentatively suggested:

$$\text{D-lactate} \rightleftharpoons \text{MG} \rightleftharpoons \text{HDP} \rightleftharpoons \text{L-lactate}$$

This scheme would explain the finding of Dakin and Dudley(15) that both D- and L-lactic acid are formed when MG is incubated with crude preparations of the liver, and may perhaps account also for the partial utilization of D-lactate in the animal body (Cori and Cori(16)).

Summary. Methylglyoxal has been incubated with rat liver extracts in the presence of kidney antiglyoxalase. During incubation, methylglyoxal and inorganic P decrease, whereas total hydrolyzable P increases. The hydrolysis curve of the Ba insoluble fraction of liver incubated with methylglyoxal resembles that obtained from hexose phosphate. Paper chromatography also gave evidence for the formation of hexose phosphate from methylglyoxal.

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Prevention of Aminonitrile Lesions in Rats with L-Triiodothyronine.* (23382)

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We have described the striking lesions of the skeleton and of other mesodermal structures produced in vertebrates by aminonitriles (1,2,3,4). The histological similarities between these experimental lesions and certain human skeletal diseases were demonstrated (5,6). The metabolism of aminoacetonitrile (AAN) in the rat was studied and the possible protective effect of many compounds against the aminonitrile lesions was tested (7,8). Recently Selye and Bois(9,10) were able to influence the severity of these lesions in rats with somatotrophic hormone and with cortisol. Selye also indicated(11) that intensive overdosage with thyroxine can suppress these lesions. The effects of l-thyroxin (Tx) and 3,5,3′ l-triiodothyronine (TRI) in rats treated with AAN are here reported.

Methods. In the first experiment (Table I) weanling male Sprague-Dawley rats weighing 42 to 50 g were fed *ad lib.* ground Purina chow containing 0.075% AAN. The animals were housed in cages in groups of 4. Four groups of rats received Tx incorporated into the AAN diet in the following proportions: 0.25, 0.50, 1.25, and 10 mg per 100 g diet, respectively. Four groups of rats received TRI incorporated into the AAN diet in the following proportions: 0.02, 0.06, 0.10, and 0.25 mg/100 g diet, respectively. Groups of 3 rats served as controls: one group was fed Purina chow alone, another group received AAN diet alone, and other groups were fed Purina chow with the same percentages of Tx and TRI as in experimental groups. The animals were weighed every 4 days. The second experiment was similar to the first except that the AAN diet contained 0.06% AAN. The doses of Tx and TRI were slightly changed (Table II). Thyroid extract 250 mg/100 g

TABLE I. Effect of L-thyroxine* (Tx) and L-triiodothyronine† (TRI) in Prevention of Skeletal Lesions in Rats Given Purina Chow Containing 0.075% of Aminoacetonitrile (AAN).

	Control diet‡		AAN diet§		
	Avg		Avg		
Treatment, mg/100 g diet	Starting wt (g)	Final wt (g)	Starting wt (g)	Final wt (g)	Severity of lesions
Purina	44	137			0
Purina + AAN 75			46	98	5+
Tx .25	44	126	42	96	5+
Tx .50	49	121	44	98	5+
Tx 1.25	47	148	48	99	5+
Tx 10 ‖	—	—	50	98	3+
TRI .02	44	132	43	100	5+
TRI .06	44	129	46	88	2+
TRI .10	48	114	48	92	1+
TRI .25¶	48	91	49	74	1+

Four rats were used to test each dose in the experimental groups; 3 rats for each control group. Duration of experiment, 17 days.

* Nutritional Biochemicals.

† "Cytomel," courtesy of Smith, Kline & French.

‡ Finely ground Purina chow. The rats on this diet and Tx or TRI did not develop skeletal lesions.

§ Finely ground Purina chow + 0.075% AAN.

‖ One rat died on 14th day of experiment.

¶ Two rats of control group and 3 of AAN group died from 11th to 13th day of experiment.

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TABLE II. Effect of L-thyroxine (Tx) and L-triiodothyronine (TRI) in Prevention of Skeletal Lesions in Rats Given Purina Chow Containing 0.06% of Aminoacetonitrile (AAN).

Treatment, mg/100 g diet	AAN diet* Avg Starting wt (g)	Final wt (g)	Severity of lesions
Purina only	54	173	0
Purina + AAN 60	59	148	3+
Tx .40	60	152	3+
1.0	64	153	3+
5.0	53	140	2+
TRI .03	62	137	1+
.06	57	130	0
.09†	61	120	0
Thyroid extr. 250	57	140	2+

* Finely ground Purina chow + 0.06% AAN.

† One rat died on 6th day of study.

of diet was added to the diet of one group of rats. In Exp. 3 (Table III) the rats were fed Purina chow containing 0.075% AAN as in the first experiment. Several doses of Tx and TRI were injected subcutaneously daily in slightly alkaline solutions. In Exp. 4 (Table IV) the daily doses of Tx and TRI were injected simultaneously with 5 mg AAN. In Exp. 5 (Table V) 2 μg TRI was injected daily simultaneously with 6 mg AAN for the first 6 days and 9 mg AAN for the following 11 days. Duration of experiments was 17 days. At termination of each experiment the animals were sacrificed and roentgenograms were made. At post mortem examination no instance of dissecting aneurysm of the aorta was noted in either control or experimental animals. The knees, including lower half of femur and upper half of tibia, were excised and decalcified in 6% aqueous solution of sulfosalicylic acid. Sagittal sections were stained with hematoxylin and eosin. Roentgenograms were evaluated for (a) width of epiphyseal plates adjoining the knees, (b) amount of periosteal new bone formation in femurs, (c) width of ilium, and (d) severity of osteoporosis. Approximate estimate of severity of lesions obtained from the roentgenograms was checked against the lesions observed in histological sections. Rats of the same experimental group showed only slight variations in severity of the lesions.

TABLE III. Effect of Injected L-thyroxine (Tx) and L-Triiodothyronine (TRI) in Prevention of Skeletal Lesions in Rats Given Purina Chow Containing 0.075% Aminoacetonitrile (AAN).

Treatment,* μg/day/rat	Avg Starting wt (g)	Final wt (g)	Severity of lesions
Control†	51	115	5+
Tx 10	48	116	5+
50	49	121	4+
100	52	116	3+
250	48	107	2+
TRI 2	49	119	2+
5	51	111	2+
10	46	95	1+

* Tx and TRI were administered in slightly alkaline solutions and inj. subcut.

† 0.1 ml normal saline.

TABLE IV. Effect of Injected L-thyroxine (Tx) and L-triiodothyronine (TRI) in Prevention of Skeletal Lesions in Rats Treated with 5 mg/Day of Aminoacetonitrile (AAN) Administered Subcutaneously.

Treatment,* μg/day/rat	Avg Starting wt (g)	Final wt (g)	Severity of lesions
Control†	48	107	3+
Tx 50	48	123	1+
100	51	130	0
TRI 2	51	145	0
5	49	118	0
10	48	116	0

* Tx and TRI were administered in slightly alkaline solutions. 5 mg AAN in 0.2 ml distilled water was inj. simultaneously.

† 5 mg AAN inj. subcut.

TABLE V. Effect of Injected L-triiodothyronine (TRI) in Prevention of Skeletal Lesions in Rats Treated with 6 mg/Day of Aminoacetonitrile (AAN) for 6 Days and 9 mg/Day AAN for Following 11 Days Administered Subcutaneously.

Treatment	Avg Starting wt (g)	Final wt (g)	Severity of lesions
Control *	52	165	0
TRI †	50	161	0
AAN ‡	51	144	4+
AAN + TRI§	51	142	0

Six rats were used in each group. Purina chow was fed *ad lib.*

* 0.2 ml normal saline inj. subcut. daily.

† TRI 2 μg administered daily in 0.2 ml slightly alkaline solution.

‡ AAN 6 mg/day in 0.2 ml distilled water for 6 days and AAN 9 mg/day in 0.3 ml distilled water for following 11 days inj. subcut.

§ TRI 2 μg was inj. daily simultaneously with AAN 6 mg/day the first 6 days and AAN 9 mg/day for following 11 days.

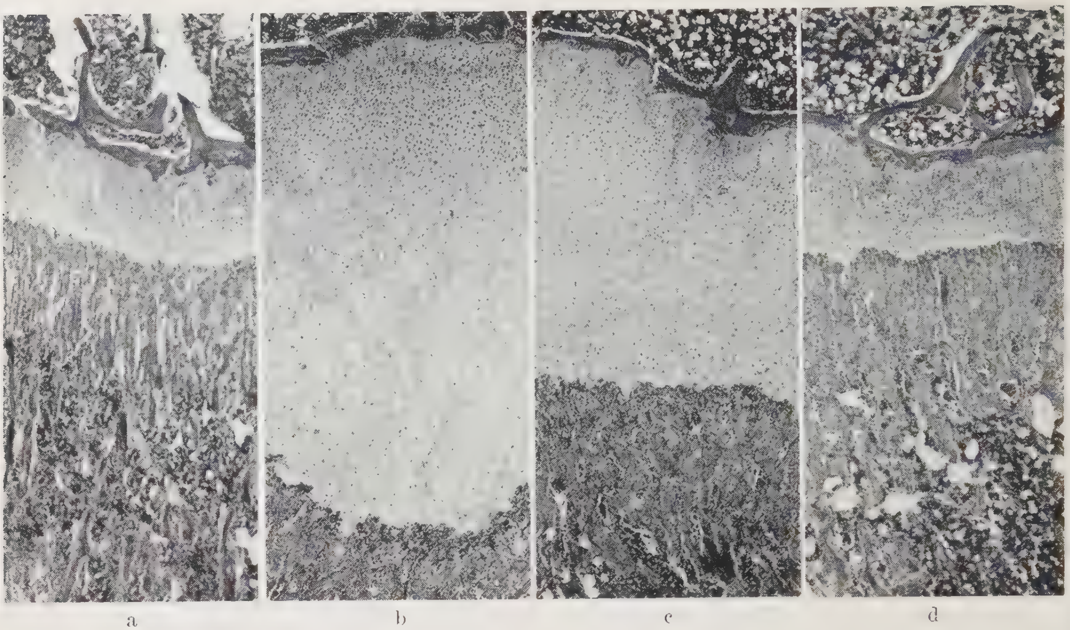

FIG. 1. Proximal epiphyseal plates of tibia of 40-day-old rats (×20, hematoxylin and eosin stain) treated as follows for 17 days: (a) normal control, (b) AAN 0.075% diet, (c) AAN 0.075% diet + Tx 100 μg/day inj. subcut., (d) AAN 0.075% diet + TRI 10 μg/day inj. subcut.

Results. Neither Tx nor TRI completely prevented the lesions produced by a relatively high dose of AAN (0.075% of diet, Tables I and III). However, the lesions were greatly improved with high levels of TRI, whereas Tx had only a slightly suppressing effect even in doses 20 to 100 times higher than TRI (Fig. 1). Less severe lesions were produced with diet containing 0.06% AAN (Table II). TRI 0.09 and 0.06 mg/100 g diet prevented the lesions; these were not prevented with Tx in doses up to 5 mg/100 g diet. Moderately severe lesions were produced with AAN (5 mg/day) injected subcutaneously; TRI (2 μg/day) injected subcutaneously prevented the lesions; they were also prevented with parenteral administration 100 μg/day of Tx, but not with 50 μg/day of Tx (Table III). The experimental animals receiving very high doses of Tx and TRI grew less than the controls on AAN alone. The objection may be raised that the suppression of the AAN lesions by these compounds is related to the growth inhibition and is non-specific. In Exp. 5 (Table V) the growth inhibition produced by AAN was not incremented by administration of TRI 2 μg daily; however, this small dose checked completely the AAN lesions. Also the experimental animals of Exp. 4 (Table IV) weighed more than the controls, yet showed complete suppression of the AAN lesions. It appears evident from these experiments that Tx and TRI can suppress the lesions produced by moderate doses of AAN without interfering with growth of the rat.

No skeletal lesions occurred in the control groups of rats fed Purina chow with Tx and TRI or in the controls injected with Tx and TRI (not included in tables). However, animals on the highest doses of Tx (250 μg/day) and TRI (10 μg/day and 0.10 and 0.25 mg/100 g diet) had epiphyseal plates slightly narrower than the normal animals with fewer cartilage cells indicating some retardation of growth (Fig. 2). It is known that thyroxin produces precocious skeletal maturation in rats(12). Several investigators have estimated, on the bases of different criteria, the potency of TRI to be about 5 times that of Tx(13,14). Working on growth and development of embryonic chick limb-bones in tissue culture Fell and Mellanby(15) found

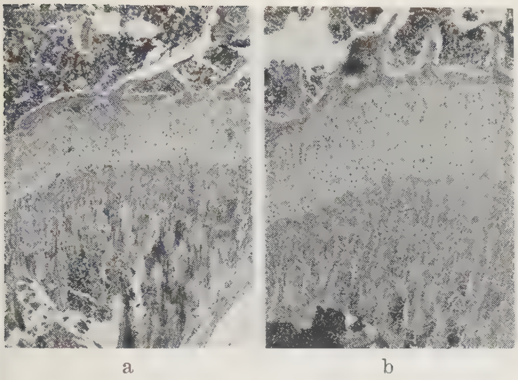

FIG. 2. Proximal epiphyseal plates of tibia of 40-day-old rats (×20, hematoxylin and eosin stain) treated as follows for 17 days: (a) TRI 10 μg/day inj. subcut., (b) Tx 100 μg/day inj. subcut.

that the inhibitory effect of TRI on growth rates of femora and tibiae was about 4 times that of Tx. Our experiments with prevention of AAN lesions in rats showed TRI to be over 100 times more active than Tx when administered orally. It is known that Tx is less than one-half as active when administered orally as when administered subcutaneously, whereas TRI is almost equally effective by either route(13,14). The results of our third and fourth experiments indicate that when administered parenterally TRI was over 50 times more potent than Tx in the suppression of the AAN lesions.

No qualitative differences could be observed between effects of TRI and Tx in rats treated with AAN. Punched out areas filled with very cellular connective tisue were observed in the long bones of AAN-treated rats on the larger doses of TRI (10 μg/day, and 0.10 and 0.25 mg/100 g diet); similar lesions were observed in Tx 250 μg/day AAN-treated rats. More than half of the animals on TRI 0.25 mg/100 g diet died of unknown cause.

The suppression of the AAN epiphyseal plate lesions with Tx and TRI may be related to the skeletal maturation effects of these compounds. However, the much greater activity of TRI on these lesions cannot be explained. Experiments now under way indicate that the beneficial action of TRI on the AAN lesions is not limited to the skeleton but is a generalized effect on all mesodermal tissues. The possible interrelation between AAN action and thyroid secretion is suggested by our observation that aminonitriles become effective in developing vertebrates at a period corresponding closely with onset of activity of the thyroid gland(16), *i.e.* the 16th day of gestation for the rat embryo(17), the 13th day of hatching for the chick embryo(4) and stage 25 for the frog larva(4).

Summary. Low doses of l-triiodothyronine suppressed the mild forms of aminoacetonitrile poisoning in rats, whereas extremely high doses of thyroxine were necessary to accomplish the same result. On the basis of the prevention of aminonitrile lesions in rats, l-triiodothyronine was estimated to be over 100 times more active than l-thyroxine when administered orally and over 50 times when administered parenterally.

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Actions of Chlorpromazine and of Reserpine on Spinal Reflex Activity in the Cat. (23383)

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These experiments are based on the finding that doses of chlorpromazine that do not modify conduction depress transmission in the inferior mesenteric ganglion of the cat (Krivoy, unpublished). If the synapse in the spinal cord resembles the ganglionic synapse, chlorpromazine should modify transmission in the spinal cord also. The present experiments were designed to study this possibility.

Methods. In one preparation(1), laminectomy was performed at the level of the last lumbar or first sacral segment to expose homolateral dorsal and ventral roots from the same spinal segment. The roots were mounted on electrodes. The skin edges around the incision were elevated and the trough so formed was filled with mineral oil maintained at 37°C. A second method(2) consisted of stimulating and recording through 2 pairs of electrodes applied to the same sciatic nerve, which had been cut distally. Electrical equipment and methods of recording have been described previously(3), only the AC amplifier being used in these experiments. Bright silver electrodes were used with spinal roots, whereas wick electrodes were used for sciatic nerve experiments. The dorsal root or sciatic nerve was stimulated with biphasic pulses at a frequency varying from .5-1/sec. with a stimulus duration of .1-.5 msec. Except where noted, stimulus intensity was 50-80% maximal. All stimulus parameters were maintained constant throughout any given experiment.

Decerebration was performed at the level of the superior colliculus under ether. Ether was stopped at least 2 hours before the experiment. Intact cats were anesthetized with pentobarbital sodium, 30 mg/kg i.p. Blood pressure was recorded from a carotid artery in the spinal root preparation and from the contralateral femoral artery in the sciatic nerve experiments. Cats with blood pressures below 120 mm Hg prior to drug administration were not used. Administration of reserpine or chlorpromazine was delayed for at least one hour after the surgical procedures and the placing of electrodes.

Chlorpromazine and reserpine were administered as solutions: 1. chlorpromazine, 2.5 g, ascorbic acid, .2 g, sodium bisulfite, .1 g, sodium sulfite, .1 g, sodium chloride, .6 g, and water, q.s. to 100 cc; 2. reserpine, .25 g, benzyl alcohol, 2 cc, citric acid, .25 g, propylene glycol, 10 cc, and distilled water, q.s. to 100 cc. Control experiments consisted of administration of the above solvents without active agents. In experiments to test the effect of cardiovascular collapse on spinal activity, animals were bled to death by section of one carotid artery.

Results. I. Stability of the preparation. Fig. 1 was prepared from a spinal root preparation to which only nembutal was administered. In the insert, records representative of the 2 types of preparations (sciatic nerve and spinal root) are shown. Essentially identical results were obtained in 8 experiments of this type. The monosynaptic reflexes were stable for prolonged periods of time. Although the polysynaptic reflexes were more variable, they also showed no consistent deterioration during the period of observation.

II. Anesthetized Cat. A. Chlorpromazine. Fig. 2A illustrates the data of an experiment typical of 12. Although both mono- and

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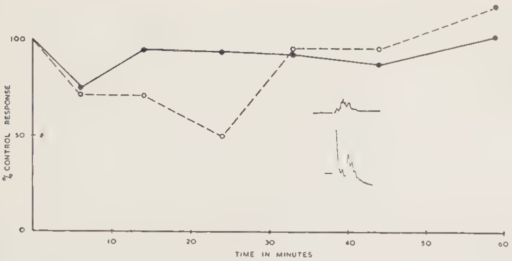

FIG. 1. Stability of monosynaptic spinal reflexes for long times. Solid line—monosynaptic activity; dotted line—polysynaptic. Data used in these graphs were from a spinal root experiment. Inserts: Upper tracing from a spinal root experiment; lower tracing from sciatic nerve preparation. Tracings used in inserts are typical. Time signal—1000 cycles.

polysynaptic activities were decreased by the drug, monosynaptic activity suffered first. The stimulus used in Fig. 2A was submaximal. When a supramaximal stimulus was used, spinal activity was modified by chlorpromazine to a considerably lesser extent. Fig. 2B shows the results obtained with supramaximal stimulation from the same preparation used for Fig. 2A. Although an effect from chlorpromazine was evident almost immediately, maximal action was not achieved for several minutes with supramaximal stimulation. The *minimal dose* of chlorpromazine necessary to decrease the response of the spinal root preparation was approximately .2 mg/kg intramuscularly or intravascularly. When the sciatic nerve preparation was used, the dose required was approximately 2-3 mg/kg. *B. Reserpine.* Results obtained with reserpine were essentially the same as those with chlorpromazine, except that there appeared to be greater specificity for monosynaptic activity. Fig. 3, typical of 9 experiments, shows an example of the effects of reserpine. Since small doses of reserpine do not act immediately, no attempt was made to determine the minimal effective dose of this drug.

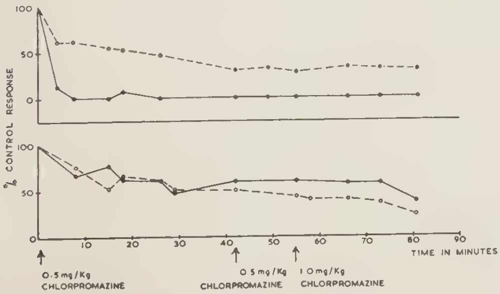

FIG. 2. Influence of chlorpromazine on mono- and polysynaptic activities in anesthetized spinal root preparation stimulated submaximally (upper graph) or supramaximally (lower graph). Solid line—monosynaptic activity; dotted line—polysynaptic.

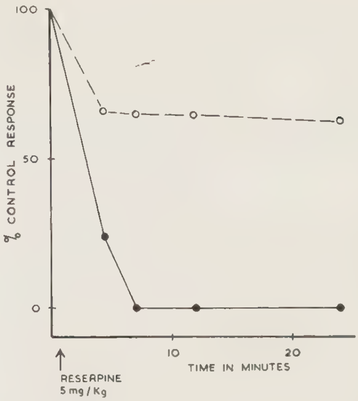

FIG. 3. Actions of reserpine on mono- and polysynaptic activities of spinal root preparation. Solid line—monosynaptic activity; dotted line—polysynaptic.

III. Decerebrate Cats. A. Chlorpromazine. Chlorpromazine in the decerebrate cat had the same action as in the anesthetized preparation, except that the specificity for monosynaptic activity was more conspicuous. Fig. 4 is typical of 11 such experiments. *B. Reserpine.* In 9 experiments, reserpine had the same actions in the decerebrate cat as those observed in the anesthetized animal. *IV. Induced cardiovascular collapse.* When cardiovascular collapse was induced by bleeding in

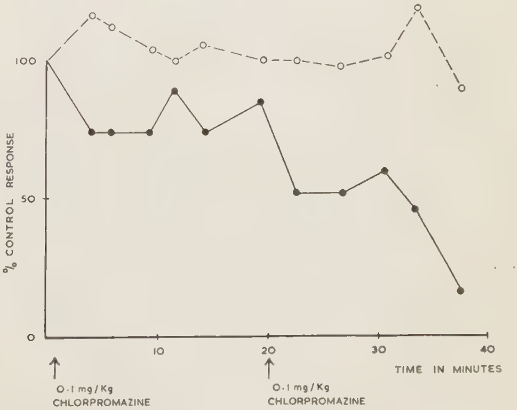

FIG. 4. Actions of chlorpromazine in the decerebrate cat. Data from a sciatic nerve preparation. Solid line—monosynaptic activity; dotted line—polysynaptic.

the absence of drugs, polysynaptic activity was depressed before monosynaptic activity was affected. In fact, monosynaptic activity frequently was enhanced as an initial response to bleeding. This information results from 6 experiments. *V. Controls.* Although the reserpine vehicle alone frequently augmented monosynaptic activity initially, neither the chlorpromazine vehicle nor the reserpine vehicle produced depression similar to those described in Section I. A total of 12 experiments was performed, 5 with the reserpine vehicle. *VI. Blood pressure.* Although both chlorpromazine and reserpine lowered blood pressure, this effect bore no temporal relationship to the actions of the drugs on the induced potentials.

Discussion. These results support the conclusions that, under the conditions of these experiments, chlorpromazine and reserpine inhibit spinal reflexes and that both have strong specificity for monosynaptic systems. Angelucci(4) has reported that both chlorpromazine and reserpine inhibit spinal reflexes in the frog; Dasgupta and Werner(5) found that chlorpromazine completely suppresses the crossed extensor reflex in decerebrate cats. On the other hand, Preston(6) has reported that doses of chlorpromazine causing obvious changes in the behavior of cats produce no observable alterations in monosynaptic and polysynaptic reflex arcs of the spinal cord; doses of 5 mg/kg of reserpine were found to increase the magnitude of the monosynaptic spike of the ventral root potential without significant change in the polysynaptic potentials(7). Our results fall between these two extremes: we find that both chlorpromazine and reserpine decrease fairly specifically the magnitude of the monosynaptic potential, which would be expected to mean that these drugs inhibit simple reflexes like the knee jerk but not more complex ones. The ultimate causes of the differences between the authors cited and between certain of these and ourselves are not apparent. Differences in technic may play an important part. If our conclusion that chlorpromazine and reserpine are relatively specific for monosynaptic reflex arcs is correct, this finding would be presumptive evidence for the existence of 2 physicochemical mechanisms in the spinal cord, one associated with monosynaptic activity and the other with polysynaptic activity. Such duality of mechanisms has been postulated by Holmstedt and Skoglund(8). These experiments support also the conclusion of Schneider(9) that reserpine leaves efferent activity practically intact even though it blocks the reflex arc. The concept that inhibition of spinal reflex activity by chlorpromazine and reserpine is a consequence of systemic cardiovascular collapse is ruled out by the finding that polysynaptic activity is more sensitive than monosynaptic to surgical shock.

Summary. 1. Chlorpromazine and reserpine inhibit spinal reflex activity. 2. The inhibition affects predominantly monosynaptic reflex pathways when submaximal stimuli and minimal doses of chlorpromazine or of reserpine are used. 3. The spinal effects of chlorpromazine and reserpine do not result from circulatory changes, unless these be local alterations in the circulation within the spinal cord. That possibility has not been studied.

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Incorporation of Glycine Into Protein Fractions of Nuclei of Liver and Hepatoma.* (23384)

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For comparison of tumor cells with corresponding normal cells it seems particularly pertinent to have information concerning content and metabolic activities of various protein fractions of the nuclei of these cells. Notable progress in fractionation of proteins of the nuclei of normal cells has been summarized by Allfrey *et al.*(1). Daly *et al.*(2) and Smellie *et al.*(3) have determined the relative incorporation of labelled glycine into several protein fractions of the nuclei of liver cells, and Allfrey *et al.*(4) have made a similar study of protein fractions of the nuclei of calf thymus cells. In the present study the proteins of the nuclei of normal liver cells of the adult rat are compared from a quantitative standpoint with corresponding fractions of the nuclei of a transplanted liver tumor. These fractions were analyzed for glycine, and the incorporation of glycine-l-C^{14} was determined.

Methods. The tumor used in these experiments originated in the liver of a rat which had been fed p-dimethylaminoazobenzene. In the fourth-transplant generation it was implanted by trocar into the axillary region on both sides of male albino rats (Carworth Farms) maintained on Purina dog chow *ad libitum*. When the tumors had attained a size of approximately 10 g, the rats, which at this time were of about adult size, were given an intraperitoneal injection of glycine-l-C^{14} (14 μc/100 g body weight) in 0.9% saline. Twenty hours thereafter the rats were sacrificed, and the livers and tumors were removed quickly for the isolation of nuclei by the method of Hogeboom *et al.*(5). The total cytoplasmic fractions of the homogenates of liver and tumor were used for isolation of samples of the total cytoplasmic proteins. An outline of the procedure used for fractionation of the proteins of the nuclei is given in Fig. 1 and 2. Details of the various steps in the procedure are presented below by the numbers with which they are designated in the figures. Procedures used in more than one step in the fractionation are described only once in the text, and the numbers are repeated in the figures. The majority of the steps in the fractionation were performed in a cold room. Unless specified otherwise, centrifugations were at a speed of 2,000 r.p.m. with the horizontal yoke No. 269 of an International refrigerated centrifuge, model PR-1. (1) Nuclei isolated from liver or tumor were suspended in 10 ml of cold 0.05 M sodium citrate (pH 7) and were homogenized in a cold

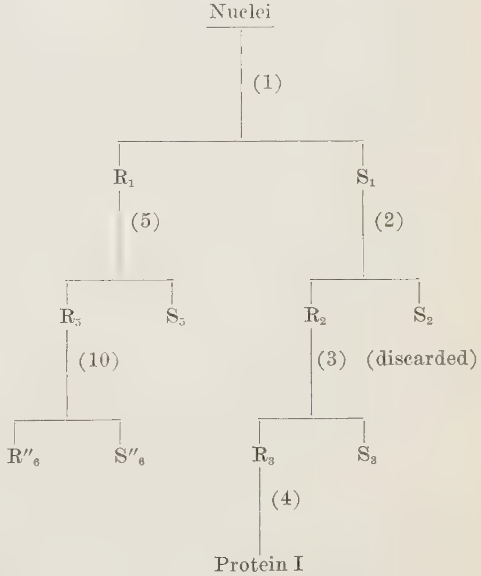

FIG. 1. Outline of procedure used for fractionation of proteins of nuclei. Numbers enclosed in parentheses refer to steps in the fractionation described in the text. S and R refer to supernatant fluid and residue, respectively.

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[†] Public Health Service Research Fellow of the Nat. Cancer Inst., 1954-1955.

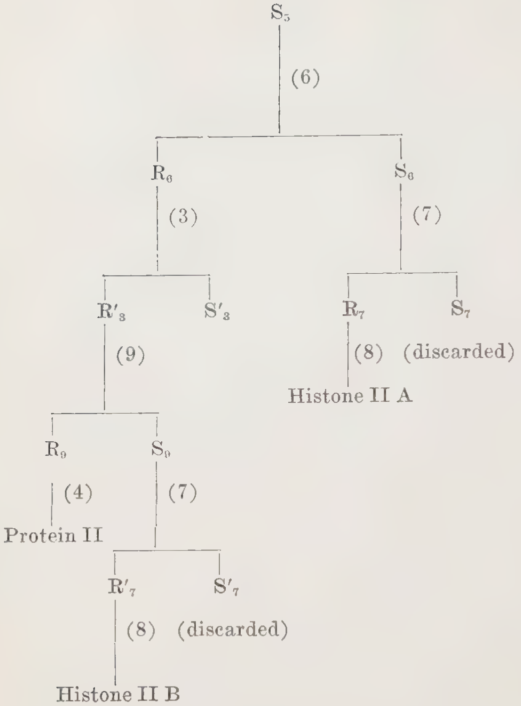

FIG. 2. Outline of procedure used in the further fractionation of S_5. Procedures for further fractionation of R''_6 and S''_6 (Fig. 1) were similar to those for R_6 and S_6, respectively.

micro-cup of a Waring blendor for 1 minute, with repetition of the process until microscopic observation revealed that the majority of the nuclei were fragmented. The homogenate was centrifuged for 30 minutes at 10,000 x G in the multispeed rotor of an International refrigerated centrifuge. The residue was suspended in a second 10 ml portion of cold 0.05 M sodium citrate, and the homogenization and centrifugation were repeated. The remaining residue (R_1) was used in step 5, and the pooled supernatant centrifugates (S_1) were treated according to step 2. (2) Cold 1 N perchloric acid was added with stirring to S_1 to yield a final concentration of 0.1 N. After 30 minutes at 5°, the precipitate was collected by centrifugation, and it was washed 3 times with 5 ml portions of cold 0.1 N $HClO_4$. (3) Residue R_2 was heated for 20 minutes at 90° in 5 ml of 1 N $HClO_4$ in order to hydrolyze and to extract the nucleic acids(6,7). After centrifugation, the residue was washed twice with small portions of 1 N $HClO_4$. The perchloric acid extract and the washings were pooled (S_3) for nucleic acid analyses(6). (4) Protein residue R_3 was washed thrice with absolute alcohol, twice with alcohol-ether (3:1) at 40°, once with ether, and was dried in a vacuum desiccator. (5) R_1 was homogenized in 10 ml of 1 M sodium chloride. The homogenate was agitated in a mechanical rocker for 6 hours at 5°, and was centrifuged at 10,000 x G for 30 minutes. (6) Hydrochloric acid was added, with constant stirring, to S_5 until a final concentration of 0.1 N was attained, and the resulting suspension stirred slowly at 25° for 2 hours. The suspension was centrifuged. (7) S_6 was brought to pH 11 with ammonium hydroxide, and 2 volumes of alcohol were added. The histone fraction R_7 was collected by centrifugation and was washed twice with 75% alcohol. (8) R_7 was purified by extraction with 5 ml of 0.004 N HCl, followed by a second precipitation with ammonium hydroxide and alcohol. The resultant histone was heated with 4 ml of 1 N perchloric acid for 20 minutes at 90°, and the residue was collected by centrifugation and washed twice with 2 ml of 1 N perchloric acid. The perchloric acid extracts and washings were discarded. The residue (histones) was washed 4 times with alcohol, twice with alcohol:ether (3:1) at 40°, once with ether, and was dried in a vacuum desiccator. (9) R'_3 was extracted for 2 hours at room temperature with 5 ml of 0.004 N HCl. The suspension was centrifuged. S_9 was treated according to procedure 7 to yield a small additional quantity of histone which was purified as described in step 8. R_9 was washed and treated by procedure 4. (10) R_5 was extracted by stirring it continuously with 5 ml of 0.1 N HCl at room temperature for 2 hours and centrifuging. The supernatant, S''_6, and the residue, R''_6, were fractionated further by the procedure outlined in Fig. 2 for S_6 and R_6, respectively. There were thus obtained fractions Protein III, Histone III A, Histone III B, and S''_3 which corresponded to fractions (Fig. 2) Protein II, Histone II A, Histone II B, and S'_3, respectively.

Radioactivities of the protein samples were determined in a Tracerlab internal gas flow counter and were corrected for background

TABLE I. Specific Activities of Protein Fractions of Nuclei and Cytoplasm of Liver and of a Transplanted Liver Tumor in Rats 20 Hours after Intraperitoneal Injection of Glycine-1-C^{14}.

	Proteins of liver		Proteins of tumor	
Protein fraction	c.p.m./mg protein	c.p.m./mg prot.-glycine	c.p.m./mg protein	c.p.m./mg prot.-glycine
		× 100		× 100
Total proteins of cytoplasm	1,850	458	940	245
Nuclear fraction I	1,354	286	730	150
Idem II	1,415	367	280	79
" III	1,680	382	820	197
Nuclear histone II	288	46	510	83
Idem III	390	59.7	620	95

and for self-absorption. They are expressed as specific activities in counts per minute per mg of protein, calculated to infinite thinness. Protein samples were analyzed for amino acids by the method of Levy(8) after hydrolysis for 20 hours with 6 N HCl in a sealed tube at 110°. In each case the dinitrophenylglycine was extracted from the paper chromatograms with 5 ml of distilled water, was determined spectrophotometrically, and then was extracted from the aqueous phase with ether. The ether extracts were evaporated upon stainless steel planchets, and the specific activities were determined and were expressed as protein-glycine (Table I).

Results. The gross compositions of the nuclei from the two sources do not differ greatly. The nuclei of the liver contain 30% of protein fraction I, 3% of fraction II, 35% of fraction III and 13% of total histones; corresponding values for the tumor are 25, 4, 32, and 14%. The proteins of fraction I (prior to treatment with perchloric acid) have properties similar to those of globulins. Fraction III corresponds to the "residual chromosomes" of Allfrey *et al.*(1) and to the lipoprotein of Wang *et al.*(9) and Engbring and Laskowski(10). Histone fraction II B was small in comparison with histone fraction II A, and these fractions were combined as histone fraction II for amino acid analyses and for determination of specific activities. This also was the case with histone fractions III B and III A. Glycine contents of the proteins of liver nuclei, as % by weight, were 4.3 for fraction I, 3.5 for fraction II, 4.0 for fraction III, 5.8 for histone II and 6.0 for histone III; corresponding values for protein fractions of tumor nuclei were 4.5, 3.3, 3.9, 5.7, and 6.1. Analyses of extracts S'_3 and S''_3 indicated that they contained deoxyribonucleic acid (or degradation products thereof) in amounts which were approximately equal to the quantities of histone fractions II and III, respectively. The major portion of the ribonucleic acid (or degradation products thereof) of the nuclei was found analytically in extract S_3, but small amounts were also found in S'_3 and S''_3.

Specific activities of the protein fractions of liver and hepatoma are recorded in Table I for one of 3 experiments of this type which were carried out. The results of 2 similar experiments were in general agreement with those of Table I. The major part (90-95%) of the radioactivity of each protein fraction could be accounted for by the radioactivity of the glycine which was isolated from the protein hydrolysates by the method of Levy. However, some radioactivity also was found in the serine, and traces were present in other amino acids. Protein fraction III had the highest specific activity of the various protein fractions of the nuclei of both liver and hepatoma cells, and the activities were only slightly less than those of corresponding total cytoplasmic proteins. Protein fraction I was next in order of specific activities in the tumor, but in liver, fraction II was more active than fraction I. The histones had distinctly lower specific activities than other protein fractions in the nuclei despite the fact that the histones have higher glycine contents than the other protein fractions. It should be pointed out that the histone fractions had much higher specific activities after one precipitation (step 7), but the contaminating radioactivity was removed during the purification procedure (step 8). Metabolic heterogeneity of the histones was indicated by the differences in the specific activities of the histone fractions in both liver and hepatoma. Each of the protein fractions of the nuclei of the liver cells had higher specific activities than corresponding protein fractions of the tumor with the notable exception of the histones which were in the reverse relationship. This difference is most readily seen in the ratios of the specific activities of protein-glycine in various protein fractions of the tumor to those of corresponding fractions of liver. These ratios are: total cytoplasmic proteins, 0.54; nuclear fraction I, 0.53; nuclear fraction II, 0.22; nuclear fraction III, 0.52; nuclear histone II, 1.8; nuclear histone III, 1.6. It is not surprising that labelled glycine was incorporated to a greater degree into most of the protein fractions of liver than into corresponding fractions of the hepatoma since the transport of the intraperitoneally injected amino acid to the liver probably would be more favorable than the transport to the subcutaneously transplanted hepatoma. However, the fact that the histones were in the opposite relationship to the other proteins and had higher specific activities in the tumor than in the liver was unexpected, and this observation is of considerable interest. These data suggest that the histones may play a particularly important role in the nuclei of the hepatoma cells. However, further work will be required to determine whether this relationship is characteristic of tumors or whether it is fundamentally characteristic of rapidly dividing cells as contrasted with non-dividing or slowly dividing cells.

Summary. A procedure is described for the separation of the proteins of cell nuclei into several fractions which differ in solubilities and in glycine content. Twenty hours after intraperitoneal injection of glycine-1-C^{14} into adult rats bearing subcutaneously transplanted hepatomas the specific activities of the protein fractions of the nuclei of liver and tumor were in the descending order: residual lipoprotein $>$ globulins $>$ histone fraction III $>$ histone fraction II. Each of the protein fractions of the nuclei of liver cells had higher specific activities than corresponding protein fractions of the hepatoma with the notable exception of the histones which were in the reverse relationship.

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Methods for Comparing Effects of Various Fats on Fibrinolysis.[*] (23385)

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Recently, much attention has been directed toward differing biologic effects of various common dietary fats. Ahrens(1,2), Beveridge(3,4), Bronte-Stewart(5), and others have shown in man that fatty meals of substances such as butter (a highly saturated fat

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containing only small amounts of essential fatty acids) will elevate cholesterol levels of blood whereas meals of substances such as corn oil (a relatively unsaturated fat containing a large amount of essential fatty acids) will lower them. The effect of these alterations in cholesterol levels of blood on tissues is not yet known. Maclagan and Billimoria (6), following an entirely different line of inquiry have found in *in vitro* experiments (using human plasma and various fats in test tubes and adding Russel-viper venom) that butter has a profound effect on clotting; that lard, oleomargarine, and several other fatty substances have a lesser effect; and that olive oil and several other fats have none. Keys, Buzina, Grande and Anderson found that fatty meals of butter, coconut oil and corn oil decreased siliconized tube clotting time approximately equally but that a meal of marine fish oil had a lesser effect(7).

We have produced myocardial or renal infarcts by dietary means in 10 of 17 rats given a diet containing 40% butter or lard by weight plus cholesterol, sodium cholate and thiouracil(8). None of 9 animals fed 20% corn oil with 20% Crisco (plus the same quantities of cholesterol, sodium cholate and thiouracil given to the rats receiving butter or lard) developed myocardial or renal infarcts. We are now attempting to extend these results with more animals. It is important to utilize many approaches in evaluating the biologic effect of various fatty substances. Therefore we have followed other lines of investigation and have studied *in vivo* and *in vitro* the effect of various types of fatty meals on the fibrinolytic mechanism in rabbits. The results of studies *in vivo* have been presented (9). We demonstrated in rabbits that intermittent fatty meals of butter or oleomargarine greatly increased (as compared with controls) number of thrombi that organize instead of lysing after repeated intravenous injections of standard amounts of clot. Similar fatty meals of corn oil had no effect.

In experiments reported herein 2 new approaches have been utilized. In Exp. 1, using a partially *in vitro* method, the effect in rabbits of single meals of butter, corn oil, coconut oil or water on fibrinolytic activity of blood was measured by a technic utilizing streptokinase to activate conversion of profibrinolysin (10) to fibrinolysin. In Exp. 2, we have modified this technic to study the effect by a completely *in vitro* method of various fats on streptokinase-lysis times of clotted human plasma. Results of both experiments indicate that butter significantly lengthens streptokinase-lysis time (under conditions specified) but that corn oil and coconut oil have little or no effect.

Materials and methods. Exp. 1. Ninety-two young adult rabbits (New Zealand White) of both sexes, weighing 2.5 kg or more were used. They were offered Purina rabbit pellets daily and water *ad libitum*. A tube was passed transorally into the stomach of each rabbit and 25 ml of either butter, corn oil, coconut oil or water introduced. Three hours later, 25 to 30 ml of blood were obtained from each rabbit by cardiac puncture and placed in a tube containing 2 mg of potassium oxalate per ml of blood to prevent clotting.

Briefly stated, the test‡ used for lytic activity consisted of adding to bovine thrombin and streptokinase a portion of oxalated blood and mixing thoroughly. The thrombin rapidly produced a clot which the streptokinase lysed presumably by activating profibrinolysin. Time required for lysis was recorded for each sample and average lysis times in various groups (butter-fed, water-fed, etc.) were compared.

Details of the procedure are: 5.5 units of bovine thrombin were used for each ml of oxalated blood; under our conditions, this amount clotted the blood in less than 30 seconds. Quantities of streptokinase varied because vials differed in activity making it necessary to alter the amount when new ones were required. However, animals were so arranged that streptokinase from each vial was used for all groups (butter-fed, water-fed, corn oil-fed). Thus variations in vials were distributed and not concentrated in a single group. The amount of streptokinase to be used was determined by preliminary testing and was that quantity necessary to produce lysis within 10 to 15 minutes in random blood samples from the water-fed group. In most tests 325 units were employed but in one vial only one-tenth of this amount was required. Nine ml of blood were used in most tests although in some only half this amount, and thrombin and streptokinase were reduced accordingly. All samples were numbered so that the examiner did not know the groups from which each came until after lysis times had been recorded. After the blood had been mixed with streptokinase and thrombin in 19 mm tubes, test tubes containing the clots were placed in a water bath at 37° and examined every 2 or 3 minutes by holding the tube up to a light. Each time the clot was examined, the tube was rotated and after lysis began it was also shaken to insure thorough mixing. The clot slowly divided into small clumps which eventually became fluid. "Lysis time" in this study was the time that elapsed between clotting and the return of blood to a fluid state containing particles not larger than one millimeter. A few samples from all groups failed to clot (or lyse) at all and were discarded.

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‡ This test was suggested to us by Dr. Sol Sherry, Professor of Medicine at Washington University. Dr. Sherry also provided us with a purified streptokinase preparation made by Lederle Laboratories. Bovine thrombin used was a commercial preparation sold by Parke Davis.

Exp. 2. For the second experiment 5 ml of blood were obtained by venipuncture from 46 healthy individuals. Their average age was 33 years (range 23 to 51), and most (88%) were males. Blood was placed immediately into tubes containing 2 mg of potassium oxalate for each ml to prevent clotting. Erythrocytes were allowed to settle or were centrifuged down and the plasma removed. Subsequent procedures were carried out with plasma rather than with whole blood (which was used in Exp. 1). Plasma from each individual was divided into 4 samples of 0.45 ml each (for studies with butter, corn oil, coconut oil, and saline). Amount of bovine thrombin and streptokinase to be used was determined by preliminary testing of each vial as described in Exp. 1. In general, amount of thrombin used was that required to produce clotting in 30 to 40 seconds, and enough streptokinase was added to cause lysis of the clot within 30 to 50 seconds in samples of plasma to which only saline was added. Plasma from each individual was tested with all 4 substances (butter, etc.) concurrently, using identical amounts of thrombin and streptokinase.

Fats to be tested were commercial products (unsalted butter,§ corn oil|| and coconut oil¶) obtained from retail firms. A small sample of each was placed in individual tubes in a 37°C water bath for one-half hour before being tested along with a tube containing saline. Butter began to melt at approximately 29°C, while other substances were, of course, in a liquid state even at room temperature. The melted butter separated into a clear supernatant layer and a turbid layer which were mixed by shaking before using. Equal amounts of the various fats and saline were used for each test. In half, this amount was 0.2 but in the other half only 0.05 ml was used.

Briefly stated, the test for the effect of the various substances on fibrinolysis was: appropriate quantities (as described above) of bovine thrombin and streptokinase were placed in 4 test tubes. Butter, corn oil, coconut oil, or saline in equal amounts were added. Plasma (0.45 ml) was added to each tube using a blow-out pipette, the mixture shaken vigorously and placed in the water bath at 37°C. As soon as the mixture appeared to be in a gel state, clotting was considered complete. After the clot had formed, the tube was removed from the water bath every 5 or 6 seconds for shaking and naked-eye inspection with a light behind the tube. Part of the clot rapidly liquified and the remaining solid portion gradually disintegrated. "Lysis time" in this study was that number of seconds measured by a stop-watch for the clot to become completely liquid except for residual threads or minute particles which persisted indefinitely.

Results. Exp. 1. Average lysis times are given in Table I for all 92 rabbits. Although individual variations occurred, average time

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§ Sealtest

|| Mazola Oil.

¶ Magnus, Maybee & Reynard.

TABLE I. Average Clot-Lysis Times (Streptokinase Method) for Several Groups of Rabbits Given Single Meals of Butter, Corn Oil, Coconut Oil or Water.

Type of meal	No. of animals in group	Avg lysis times (min.)	Stand. error of mean
Butter	31	22.0	1.47
Water	23	15.0	1.53
Corn oil	16	15.5	2.63
Coconut oil	22	13.0	1.62

Avg lysis time for the group fed butter is significantly longer than that for any other group. Differences in lysis time among the other groups are not statistically significant.

for the group that received butter was 22 minutes, significantly higher than that for any other group (using the t test p = 0.01 for comparisons between butter and water and between butter and coconut oil; p = 0.05 for comparison between butter and corn oil). Average lysis times for the groups receiving corn oil (15.5 min) and coconut oil (13 min) do not differ significantly from that of the group receiving water (15 min). Plasma from rabbits given fatty meals did not appear fatty. Plasma cholesterol levels of many samples were never above 116 mg%.

Exp. 2. Results are presented in Tables II and III. Average lysis time in seconds for samples of human plasma containing 0.2 ml of the various substances were: butter 158, corn oil 68, coconut oil 60, saline 43. Corresponding times when 0.05 ml of lipids or saline were added were: butter 176, corn oil 68, coconut oil 63, saline 37. With either 0.05 or 0.2 ml of fat added, butter greatly lengthens lysis time as compared with all other substances tested (p = 0.01 as determined by the t test). The minimum amount of any fat tested that would be required to produce a significant lengthening of the lysis time was not determined; in each case, however, it was found that 0.05 ml of lipid produced as great an effect as 0.2 ml. Lysis time of human plasma with corn oil and coconut oil was significantly longer than that with saline with either 0.2 or 0.05 ml of lipid added (using the t test p = 0.01 for comparisons with saline and corn oil; for comparison with saline and coconut oil p = 0.01 with 0.05 ml added and p = 0.05 with 0.2 ml added). Difference in lysis times using corn oil and coconut oil was not significant (p = 0.1 with both 0.2 ml and 0.05 ml added).

TABLE II. Average Streptokinase-Lysis Times of Clotted Human Plasma after Addition *In Vitro* of 0.2 ml of Various Fats to 0.45 ml of Plasma.

Substances tested	No. of samples	Avg lysis time (sec.)	Stand. error
Saline	23	43	5.6
Butter	23	158	12.1
Corn oil	23	58	4.9
Coconut oil	23	60	6.2

Lysis times for butter, corn oil and coconut oil are significantly longer than that for saline. Difference in lysis times between corn oil and coconut oil is not significant but differences between butter and corn oil and between butter and coconut oil are.

TABLE III. Average Streptokinase-Lysis Times of Clotted Human Plasma after Addition *In Vitro* of 0.05 ml of Various Fats to 0.45 ml of Plasma.

Substances tested	No. of samples	Avg lysis time (sec.)	Stand. error
Saline	23	37	2.1
Butter	23	176	16.7
Corn oil	23	68	6.8
Coconut oil	23	63	4.5

Lysis times for butter, corn oil and coconut oil are significantly longer than that for saline. Difference in lysis times between corn oil and coconut oil is not significant but differences between butter and corn oil and between butter and coconut oil are.

Discussion. Our experiments provide further evidence that various fats (butter, corn oil, coconut oil) differ in their biologic effects. The significance of differences reported by others and herein in relation to development of arteriosclerosis and thrombosis in man is not yet clear. Broad conclusions and recommendations are not warranted. However, in view of possible implications for man, some tentative conclusions are suggested.

It must be emphasized that procedures so far used to evaluate the biologic effects of various fats are highly artificial. Also, although butter and corn oil have been studied most extensively, other fatty substances may have similar biologic effects or at least differ only in degree (for example, the coconut oil used here). Further, butter, corn oil and other commonly used fats are actually complex mixtures of many fatty substances. Additional investigation may disclose that highly specific constituents (that can be added or removed at will) are responsible for the differing biologic effects. At least in the experiments reported herein differences cannot be simply a reflection of degree of saturation since coconut oil (highly saturated) had no demonstrable effect whereas butter, (another highly saturated fat) had a significant effect.

Definitive conclusions cannot now be drawn but facts thus far discovered cannot be ignored. At least 5 different approaches have been utilized in comparing biologic effects of various fats: (1) on cholesterol levels in man (1-5); (2) on clotting of human blood *in vitro* (6); (3) on production of myocardial infarcts in rats by dietary means(8); (4) on thromboembolic-induced pulmonary arterial lesions in rabbits(9); (5) on fibrinolytic activity of blood of rabbits and man (reported herein). In every instance butter and similar substances have had an "undesirable" effect whereas in most instances corn oil and similar substances have had either a negative or a "desirable" effect. We believe available information regarding differences in biologic effects of fats such as butter and corn oil is impressive enough to make imperative an immediate, concentrated effort to obtain definitive data regarding the importance of the differences.

Our interest in the effect of a substance on the fibrinolytic system stems from the fact that the precipitating cause of death or disability in individuals with arteriosclerosis is usually thrombosis. If it could be prevented or if thrombi once formed could be lysed rapidly, victims of arteriosclerosis might live quite satisfactorily with their lesions. Thrombosis seldom occurs in normal vessels with smooth linings but frequently occurs in roughened arteriosclerotic vessels. The body has a defense mechanism in the form of a fibrinolytic system for rapidly lysing thrombi. It is obvious that factors influencing this system are exceedingly important, particularly to individuals with arteriosclerotic plaques.

Summary. In experiments reported herein, 2 new methods have been utilized to compare the biologic effect of 3 dietary fats. (1) In Exp. 1 a study has been made in rabbits of the effect of single meals of butter, corn oil, coconut oil and water on fibrinolytic activity of blood (drawn 3 hours after feeding and measured by a technic utilizing streptokinase to activate profibrinolysin) *in vitro* under controlled conditions. Lysis time so measured was significantly prolonged in rabbits fed butter as compared with those fed either corn oil, coconut oil or water. Meals of corn oil and coconut oil had no more effect on lysis time than did water. (2) In Exp. 2 the effect of butter, corn oil, coconut oil and saline added *in vitro* on fibrinolytic activity of clotted *human plasma* was measured by a technic utilizing streptokinase to activate profibrinolysin under controlled conditions. Under these conditions butter markedly and significantly ($p = 0.01$) prolonged lysis time of human plasma, whereas corn oil and coconut oil had a lesser effect. (3) Other substances and approaches must be studied before definitive conclusions can be drawn regarding relationships of various fats to arteriosclerosis and thrombosis in man. Our experiments are significant in that an additional approach (which may be particularly useful for rapid screening) has been added to our armamentarium for study of biologic effects of various fats.

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Metabolic Effects of Phenethyldiguanide, A New Hypoglycemic Compound.* (23386)

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With the introduction of the sulfonylureas for treatment of diabetes mellitus, renewed attention has been paid to the use of orally effective compounds. The sulfonylureas have proven to be useful in selected diabetic patients, but in a significant number of subjects they have failed to control the metabolic imbalance. Therefore, a considerable effort has been made to find other compounds which might be more effective than the sulfonylureas. In a search for such compounds, Ungar (1) recently discovered that phenethyldiguanide (PEDG), administered orally or parenterally, caused profound hypoglycemia in certain animals. This interesting observation prompted investigation of the mechanism by which hypoglycemia is produced. An understanding of the mode of action of PEDG would be of great help in its clinical applications. In an attempt to elucidate this problem, we have made studies of the effect of PEDG on glucose, glycogen, lactic acid, urea and non-protein-nitrogen *in vivo* and *in vitro*.

Materials and methods. Guinea pigs weighing between 450 and 750 g were used in these studies because they have been shown to be more sensitive to PEDG† than other animal species(1,2). In each type of experiment at least 24 animals were sacrificed, 12 serving as experimentals and 12 as controls. The drug was injected subcutaneously because responses after administration by this route were more uniform than after oral or intraperitoneal intake. The dose used was 20 mg/kg.

The following determinations were made 4 hours after PEDG administration as well as at longer and shorter intervals: blood glucose by the method of Somogyi(3), liver and muscle glycogen, using KOH for tissue digestion by the anthrone reagent of Carroll *et al.*(4), blood urea nitrogen by the method of Barker (5) and blood lactic acid by the method of Barker and Summerson(6). Non-protein-nitrogen in the total amount of urine produced during the 4 hours following PEDG injection was determined by the method of Fee *et al.*(7). In order to collect all of the urine, a small abdominal incision was made, the bladder emptied and the urethra ligated just before PEDG was given. Four hours later the urine in the bladder was collected with a needle and syringe.

The first part of the study *in vitro* was carried out on guinea pig liver slices. Slices weighing approximately 100 mg were incubated for 45 minutes at 37° in 2 ml of a solution containing 4 parts of 0.9% NaCl and one part of 0.1 M potassium phosphate buffer, pH 7.5. The beakers were placed in a Dubnoff apparatus and shaken at a rate of 110 oscillations per minute. Three different concentrations of PEDG were tested, at 50, 250 and 500 μg per ml of medium. At the end of the incubation, glucose and lactic acid concentration in the medium and glycogen concentration in the tissue were measured, using the same chemical methods mentioned in the *in vivo* studies.

The second part of the *in vitro* study was conducted with isolated rat diaphragms.

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† PEDG has been supplied through the courtesy of Dr. Louis Freedman, U. S. Vitamin Corp., Yonkers, N. Y.

TABLE I. Effect of PEDG on Blood Glucose, on Liver and Muscle Glycogen.

	Blood glucose, mg %	Liver glycogen, mg/g		Muscle glycogen, mg/g	
		Non-fasted	Fasted	Non-fasted	Fasted
Experimentals	31 ± 4*(12)†	.6 ± .06(19)	.3 ± .01(19)	7.3 ± .4 (11)	5.5 ± .1 (20)
Controls	103 ± 7 (14)	20.5 ±5.4 (18)	2.0 ± .4 (18)	10.7 ± .2 (11)	5.4 ± .1 (21)
p	<.001	<.001	<.001	<.001	

* Stand. error of mean. † No. of animals.

Hemidiaphragms, weighing approximately 125 mg were incubated in 2 ml of Gey and Gey bicarbonate buffer containing 300 mg glucose/100 ml. One-half of the hemidiaphragm served as a control, while to the other half, 500 μg PEDG/ml was added. The Warburg flasks were flushed for 5 minutes with 95% O_2 and 5% CO_2. After incubation of the hemidiaphragms at 37° for 90 minutes, glucose and lactic acid concentration in the medium and glycogen concentration in the tissue were determined. In the experiments where oxygen consumption was measured, the hemidiaphragms were incubated in a potassium phosphate sodium chloride buffer solution and the flasks were flushed with air or with oxygen.

Results. It was found that the hypoglycemia caused by PEDG was accompanied, in fed as well as in fasted animals, by a rapid fall in glycogen content of the liver. This fall was readily demonstrable 2 hours after PEDG injection but was most marked after 4 hours (Table I). Muscle glycogen determinations made at several time intervals failed to show any difference in concentration between control and experimental groups if the animals were fasted for 24 hours prior to the injection. In fed animals there was a slight but significant drop in glycogen concentration 4 hours after injection (Table I).

Table II indicates that the non-protein-nitrogen excretion in the urine which was collected during the 4 hours following PEDG administration, was markedly lower than in the controls. Blood urea nitrogen was slightly elevated by PEDG treatment. In order to eliminate the effect of PEDG on the kidney, blood urea nitrogen was determined in nephrectomized animals. Table II shows that in such animals PEDG was associated with less rise in blood urea nitrogen concentration.

PEDG caused a rise in blood lactic acid. (Table III). This rise was observed one hour after PEDG injection and even before the blood glucose started to drop, and was also evident 4 hours after PEDG administration and was even more marked in nephrectomized animals.

Addition of PEDG to the incubation medium of guinea pig liver slices caused a small but significant rise in lactic acid production. There was a decrease in glycogen concentration in the tissue but no significant increase in glucose output (Table IV). (These effects are obtained with a concentration of 250 μg PEDG per ml incubation medium, but they are more pronounced if 500 μg per ml is added.)

In isolated rat diaphragms, PEDG in a concentration of 500 μg per ml caused an increase in lactic acid formation, an increased glucose uptake, a decreased glycogen formation, and a decreased oxygen consumption (Table V).

TABLE II. Effect of PEDG on Nitrogen Metabolism.

	4 hr urinary NPN excretion, mg	Blood urea nitrogen, mg %	Blood urea nitrogen, mg % Nephrectomized animals
Experimentals	12.5 ± 2.4*(9)†	31.8 ± 5.4 (16)	42 ± 2.8 (11)
Controls	39.2 ± 14.6 (9)	20.1 ± 7.0 (15)	64.6 ± 2.6 (11)
p	<.01	<.01	<.001

* Stand. error of mean. † No. of animals.

TABLE III. Effect of PEDG on Blood Lactic Acid (mg %).

	1 hr	4 hr	4 hr Nephrectomized animals
Experimentals	20.7 ± 1.7*(10)†	27.3 ± 2.3 (9)	75.6 ± 3.5 (6)
Controls	13.5 ± .3 (10)	15.8 ± 1.5 (10)	14.1 ± 3.1 (6)
p	<.01	<.001	<.001

* Stand. error of mean. † No. of animals.

Discussion. The observation that PEDG causes an early and marked decrease in liver glycogen concentration rules out the possibility that hypoglycemia is produced by a decreased glycogenolysis in the liver. The fact that no rise in muscle glycogen concentration is observed after PEDG administration indicates that hypoglycemia cannot be explained on the basis of an increased glycogen deposition in the muscle. Williams *et al.* reported that $C^{14}O_2$ production is not significantly different in either PEDG treated or control animals following administration of glucose-U-C^{14}(8), suggesting that hypoglycemia is not produced by an increased glucose oxidation.

Since there was evidence that PEDG did not cause an increased glucose utilization, the possibility of a decreased gluconeogenesis was then investigated. It was apparent from the studies on nitrogen metabolism that PEDG decreases urea formation. A decreased gluconeogenesis from protein is consistent with such a finding and may contribute significantly to the hypoglycemic action of PEDG.

It is likely, however, that this is not the only mechanism by which hypoglycemia is produced. It was found, indeed, that PEDG had a marked effect on lactic acid production. An increased lactic acid formation was observed in the total guinea pig, in surviving guinea pig liver slices, and in isolated rat diaphragm. These changes suggest that an increased anaerobic glycolysis might also account for the hypoglycemic action of PEDG. The increased glucose uptake, decreased glycogen deposition and oxygen consumption in the diaphragms and the decrease in liver glycogen concentration observed *in vivo* and *in vitro* are consistent with such a hypothesis.

It is evident from the data presented that PEDG acts like Synthalin A in many respects, but is different from insulin and the sulfonylureas.

Summary. When phenethyldiguanide (PEDG) is administered to guinea pigs, it produces hypoglycemia accompanied by depletion of liver glycogen with no increase in muscle glycogen. PEDG causes decreased

TABLE IV. Effect of PEDG on Glucose and Lactic Acid Output and on Glycogen in Guinea Pig Liver Slices.

	Glucose output, mg/g	Lactic acid output, mg/g	Glycogen at end of incubation, mg/g
Experimentals	11.7 ± 7.9*(13)†	1.14 ± .09 (13)	39 ± 2.9 (7)
Controls	10.5 ± 6.0 (13)	.65 ± .04 (13)	47 ± 2.0 (7)
p	<.5	<.001	<.05

* Stand. error of mean.

† No. of liver slices. Not more than 6 slices (3 controls - 3 experimentals) were made out of one liver.

TABLE V. Effects of PEDG on Glucose Uptake, Lactic Acid Output, Oxygen Uptake, and Glycogen in Isolated Rat Diaphragms.

	Glucose uptake, mg/g	Lactic acid output, mg/g	Glycogen at end of incubation, mg/g	Oxygen uptake, μl/g
Experimentals	8.1 ± .6*(12)†	5.3 ± .5 (12)	1.4 ± .3 (12)	1330 ± 93 (8)
Controls	5.6 ± .5 (12)	2.0 ± .3 (12)	2.8 ± .2 (12)	1910 ± 93 (8)
p	<.01	<.001	<.001	<.001

* Stand. error of mean. † No. of hemidiaphragms.

blood urea formation and decreased non-protein nitrogen excretion in the urine. It also produces an early and significant rise in blood lactic acid. From studies made on surviving slices of guinea pig liver, it is evident that PEDG increases lactic acid formation and increases glycogen depletion, but it does not affect glucose output. PEDG also exerts an effect on isolated rat diaphragm, increasing glucose uptake, increasing lactic acid production, decreasing glycogen formation, and decreasing oxygen uptake.

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Calcification XVIII. Lack of Correlation between Calcification *in vitro* and Glycolytic Enzymes.* (23387)

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Harris(1) reported that the glycogen present in preosseous cartilage played a role in calcification. Robison and Rosenheim(2) found that certain enzyme inhibitors such as iodoacetate, cyanide and fluoride caused inhibition of calcification *in vitro*. From these observations it was suggested that a phosphatase enzyme system in preosseous cartilage was responsible for calcification. Later, Gutman(3,4) stated that one of the major difficulties of the Robison postulate was its failure adequately to demonstrate the presence of significant amounts of phosphoric ester substrate at site of phosphatase activity in the hypertrophic cartilage. Gutman then presented the hypothesis that a phosphorylative enzyme initiated the degradation of glycogen to glucose-l-phosphate which then passed through the glycolytic cycle of hexose and triose phosphates to phosphopyruvate and that one of these phosphoric esters acted as substrate for the phosphatase present in the preosseous cartilage. In a study of the effect of glycolytic enzyme inhibitors on calcification *in vitro* it was concluded that calcification was directly dependent, in some way, on glycogenolysis. More recently, Albaum, Hirshfeld and Sobel(5) reported that most of the enzymes of glycolysis present in muscle and yeast appeared to be present in epiphyseal cartilage.

The aim of this investigation was to explore the relationship that may exist between calcification *in vitro* and the glycolytic enzymes. Our previous studies, utilizing an *in vitro* calcification technic, revealed that freezing(6), heating(7) and demineralizing(8) rachitic rat tibial slices evoked inactivation of the calcifying mechanism in preosseous cartilage which could be reactivated following appropriate treatment with $CaCl_2$.

In the case of the demineralized bone it was necessary to treat first with chondroitin

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sulfate or basal salt solution at a pH of 8.2 then with $CaCl_2$ to adequately reactivate the calcifying mechanism. In the present study, enzyme activity of the preosseous cartilage and *in vitro* calcification studies on similarly treated bones are reported.

Materials and methods. Twenty-five to 30 rats of an inbred Wistar strain were used in each experiment. The animals were weaned after 3 weeks and placed on a rachitogenic diet consisting of 75% degerminated corn meal, 19% wheat gluten, 2% brewer's yeast, 3% calcium carbonate and 1% sodium chloride. After 3 to 4 weeks on the diet, the animals were sacrificed, the tibiae excised and freed of soft tissue. The proximal ends (except in the case of the whole tibiae) were sliced longitudinally, in a frontal plane, into sections approximately 1 mm in thickness. A section from each animal was immediately tested to determine if healing of the rickets had taken place. Where the silver line test showed the slightest trace of healing, the animal was discarded. In addition, a daily check of weights and appearance of the animals was made and if an animal lost weight on 2 successive days, it was discarded.

In the *first experiment,* tibial slices from the left leg were placed without further treatment into a deep freeze chest at –25°C for 2 weeks. The slices from the right tibiae were shaken 1 hour in 150 meq/l of $CaCl_2$ solution at pH 7.2. The slices were rinsed with distilled water to remove excess Ca ions and then placed in the deep freeze chest for 2 weeks at –25°C. After this period of storage the cartilages were dissected out in the cold room and used for enzyme activity studies. Several of the untreated and Ca ion treated slices, both before and after deep freeze storage, were subjected to *in vitro* calcification studies.

In the *second experiment,* the whole tibiae from the left legs were placed in deep freeze chest for 24 days. The cartilages from the right tibiae were excised and immediately tested for enzyme activity. Two of the unfrozen tibiae were sliced and subjected to *in vitro* calcification. After 24 days the cartilages of the deep freeze stored bones were excised and studied for their enzyme activity, and simultaneously, 2 of the tibiae were sliced and used for *in vitro* calcification.

In the *third experiment,* slices for the left tibiae were shaken 1 hour in 150 meq/l of $CaCl_2$ at pH 7.2. The right tibial slices were shaken 1 hour in 150 meq/l of NaCl at pH 7.3. After rinsing with distilled water to remove excess of reagent, the slices were stored for 2 weeks in deep freeze at –25°C. The cartilages were removed and used for enzyme studies. Several slices, before and after deep freeze, were subjected to *in vitro* calcification.

In the *fourth experiment,* the cartilages of fresh, untreated tibial slices from the left legs were excised in the cold room. The slices from the right tibiae were shaken 1 hour in 150 meq/l of $CaCl_2$ at pH 7.2, rinsed in distilled water and the cartilages excised in the cold room. Both sets of cartilages were used for enzyme activity studies. Simultaneously, several treated and untreated slices were used for *in vitro* calcification.

In the *fifth experiment,* tibial slices were heated for 30 or 60 minutes in distilled water at 65°C in a thermostatically controlled water bath. One slice/10 ml of water in a 15 ml capacity pyrex test tube was used. Following this, the cartilages were excised and studied for enzyme activity. Several slices were used for *in vitro* calcification and fresh, unheated slices were used for controls.

In the *sixth experiment,* disodium ethylenediamine tetraacetic acid (EDTA) at pH 4 was used to demineralize tibial slices. Three, fresh portions of EDTA were added over a 48-hour period with intermittent agitation. Chemical analysis showed that all of the calcium had been removed from the bones. The slices were thoroughly washed with 0.04% acetic acid to remove the EDTA and divided into 4 portions. One portion, "A", was shaken slowly for 20 hours at 24-26°C in 1:10 basal salt solution (described in the second footnote) at pH 8.2. Portion "B" was shaken 20 hours in the basal salt solution, rinsed with distilled water at pH 7.4 and shaken 1 hour in 500 meq/l of $CaCl_2$ adjusted to pH 9 with dilute NaOH. Portion "C" was treated 18 hours at 5°C in 1% chondroitin sulfate solution[†] at pH 4, rinsed with distilled water at pH 7.4 and shaken 20 hours in the basal salt solution. Portion "D" received the same treatment as "C" and in addition was shaken 1 hour in 500 meq/l of $CaCl_2$ at pH 9. All slices were given a final rinse with distilled water. Several slices from each portion were used for *in vitro* calcification. Enzyme activity was determined on both the excised cartilages and the decalcified true bone tissues (epiphyseal trabeculae and diaphysis) of the "A" and "D" portions.

All *in vitro* calcification studies were carried out in an inorganic medium which was prepared by adding to a one liter volumetric flask, 100 ml of a stock basal salt solution,[‡] 50 ml of a NaH_2PO_4 - Na_2HPO_4 solution that had a phosphorus concentration of 1 mg/ml and 700 ml of double, all glass, distilled water. The pH was lowered to about 6.3 by aerating with 100% CO_2. To this solution were added 6 ml of specially purified calcium chloride solution that had a Ca ion concentration of 20 mg/ml (1000 meq/l). Thus, the *in vitro* calcifying medium had a Ca x P product of 60, *i.e.*, 12 mg% Ca and 5 mg% P. Distilled water was added to the liter mark. The pH of final solution was adjusted to 7.3 by aerating with 100% nitrogen. A model G Beckman pH meter was used.

For the *in vitro* calcification process, a tibial slice was placed in a 50 ml Erlenmeyer flask filled with the calcifying medium at pH 7.3 ± 0.5. The flask was tightly stoppered with a paraffined cork and a rubber band that was stretched doubly over the cork and under the bottom of the flask. After incubation for 18 hours in a thermostatically controlled 37°C incubator, the slice was removed with forceps and rinsed in a stream of distilled water. It was placed in a concavity of a glass spot plate, covered with 3% silver nitrate solution, exposed to light from a 100 watt tungsten filament lamp for 5 minutes, and examined with a Leitz wide field stereobinocular microscope at a magnification of 32 X. Degree of calcification *in vitro* was evaluated by height and length of the silver stain obtained in the hypertrophic, provisional zone of calcification of the epiphyseal cartilage. The grading or evaluation has been described by Sobel(9).

Activity of the different glycolytic enzymes was measured on aqueous extracts of cartilage slices or on cartilage removed from whole bones. In a few instances shafts were employed. Approximately 250 mg of tissue was ground in cold water with a glass homogenizer. The extract so obtained was filtered through fine cheese cloth to remove material that could not be homogenized and made to final volume so that each ml of final extract contained 10 mg of tissue. The different enzymes were measured by adding their specific substrates to a system in which oxidation or reduction of DPN was used as indicator of activity. For example, for measurement of lactic dehydrogenase the test system contained 0.2 ml of 0.54 M sodium pyruvate, 0.1 ml of .002 M reduced DPN, 0.3 ml of tissue extract, 0.2 ml of 0.1 M ammonium phosphate buffer pH 7.6 and water to 3 ml. On addition of the pyruvate, which was added last, the cuvette was placed in the spectrophotometer previously set at 3400 Å and decrease in density due to oxidation of DPN was measured at 15 second intervals. Since pyruvate and DPN are present in excess, rate of oxidation of DPN becomes an index of lactic dehydrogenase present in the extract. By similar procedures, using the same buffer and a final volume of 3.0 ml, 0.1 ml of 0.25 M glucose in the presence of 0.2 ml of .0007 M ATP was used to measure hexokinase activity; 0.1 ml of .0058 M glucose-6-phosphate in place of the glucose was used to measure phosphohexoisomerase activity. The substitution of 0.1 ml of .0058 M fructose-6-phosphate in place of the glucose was used as an index of phosphofructokinase activity. Two-tenths of .00016 M hexose diphosphate in place of both ATP and glucose was used to measure aldolase activity.

In this last group of determinations changes in density were recorded at 5-minute inter-

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[†] We wish to thank Prof. Erik Jorpes of the Karolinska Institut, Stockholm, Sweden for the chondroitin sulfate.

[‡] The stock basal salt solution contained 0.7 M NaCl, 0.05 M KCl and 0.22 M $NaHCO_3$/liter. It was Berkefeld filtered before use and stored in a pyrex bottle.

TABLE I. Influence of Deep Freeze and Calcium Ions on Calcification *In Vitro* of the Epiphyseal Cartilage.

Treatment prior to calcification *in vitro*	Degree of calcification *in vitro* (Ca × P = 60) Mean
a. Tibial slices stored at –25°C for 2 wk	0 (0)
b. *Idem*; shaken 1 hr in 150 meq/l $CaCl_2$	1.0 (++++)
c. Tibial slices shaken 1 hr in 150 meq/l $CaCl_2$; deep freeze stored at –25°C for 2 wk	1.0 (+++)
d. Whole tibiae stored at –25°C for 24 days	0 (0)
e. *Idem*; shaken 1 hr in 150 meq/l $CaCl_2$	1.7 (+++)
f. Whole tibiae prior to deep freeze storage	2.0 (++++)
g. Tibial slices shaken 1 hr in 150 meq/l of NaCl	0 (0)
h. *Idem*; deep freeze stored at –25°C for 2 wk	0 (0)
i. Tibial slices after NaCl and deep freeze treatments; shaken 1 hr in 150 meq/l of $CaCl_2$	1.0 (++++)
j. Fresh tibial slices (not frozen)	1.5 (++++)
k. *Idem*, shaken 1 hr in 150 meq/l of $CaCl_2$	2.0 (++++)
l. Fresh tibial slices, shaken 1 hr in 150 meq/l of NaCl; shaken 1 hr in 150 meq/l of $CaCl_2$	1.3 (++++)

vals; the tables record calculated change in density occurring/10 minute interval. Operation of this test system is based on the assumption that the system contains α glycerophosphate dehydrogenase. That such a system occurs was demonstrated previously(5). To verify the presence of this latter enzyme α glycerophosphate itself was used as a substrate.

One final enzyme assay was carried out: phosphoglyceric acid was employed to determine enolase activity. Each cuvette contained .3 ml of tissue extract, .2 ml of .00085 M ADP, .2 ml of .016 M phosphoglyceric acid, .1 ml of .002 M reduced DPN, .2 ml of .1 M ammonium phosphate buffer pH 7.6 and water to 3 ml. In one control cuvette the substrate was eliminated. In a second control cuvette .3 ml of .1 M sodium fluoride was substituted for an equivalent quantity of water. In this test system the phosphoglyceric acid is converted into phosphopyruvic acid which then contributes its phosphate to ADP, forming pyruvate. In the presence of excess lactic dehydrogenase, known to be present in cartilage extracts, the pyruvate is converted to lactate with a consequent oxidation of DPN, measured as a decrease in density at 3400 Å. Enolase activity is presumed to be present only when the change in density in presence of substrate is in excess of that occurring in its absence, and when this increase may be abolished in the presence of fluoride. Changes in density were recorded at 5-minute intervals and the rate calculated on a 10-minute basis.

Results. From Table I it is apparent that deep freeze storage inactivates the calcifying mechanism in the epiphyseal cartilage as measured by the silver line test. However, this inactivation can be reversed or prevented in the presence of Ca ions(6). Metachromasia studies provided further evidence that inactivation took place during deep freeze storage. The procedural details and the method of evaluating the degree of metachromasia have been described(10). Briefly, the deep freeze stored slices showed practically no metachromasia when soaked for 18 hours at 25-26°C in a solution of toluidine blue O containing 39 mM of the dye/l of solution. When the toluidine blue O contained Ca ion, metachromasia became evident and reached a maximum at 10 meq/l and declined to 0 at 100 meq/l. Fresh untreated controls or $CaCl_2$ treated fresh or frozen sections showed some metachromasia even in the absence of Ca ion. In all cases, the metachromasia increased with Ca ion reaching the maximum at 10 meq/l and declining to 0 at 100 meq/l. The intensity of these at the maximum was more than that of the frozen bone.

TABLE II. Glycolytic Enzymes following Various Treatments That Affect Calcifiability of Hypertrophic Preosseous Cartilage.

	Change in density, 10 min.								
	Preliminary treatment								
	Fresh untreated		Frozen§ control						
Substrate	Mean†	Range	Mean‡	Range	Fresh $CaCl_2$ treated	$CaCl_2$ treated then frozen	30 min. at 65°	EDTA	EDTA‖ + C.S.¶ + Ca ions
No substrate	3.3	1.5- 5.5	3.2	3.0- 3.4	.5, 1.2	.0, .0	.0	.0	.0
" " ATP	3.8	2.5- 5.5	3.8	3.3- 4.0	.5, 1.2	.0, .0	.0	.0	.0
Glucose "	4.2	3.0- 6.2	4.5	4.0- 5.2	.5, 1.2	.0, 1.0	1.0	.0	.0
Glucose-6-P "	5.3	3.7- 7.0	4.9	4.2- 6.0	.5, 1.0	.0, .0	.0	.0	.0
Fructose-6-P "	6.5	4.5- 10.0	6.0	5.0- 7.0	1.0, 1.2	.0, .0	.0	.0	.0
Hexose diphosphate	33.2	10.0- 69.0	27.7	20.0- 42.0	.0, 1.0	.0, .0	.0	.0	.0
Glycerophosphate	19.8	7.5- 42.0	28.2	21.0- 36.0	.0, 1.0	.0, .0	.0	.0	.0
Phosphoglyceric acid	9.7	6.0- 16.0	9.3	7.0- 12.0	5.8, 1.8	2.0, 5.0	.0	.0	.0
Pyruvic acid*	158.3	120.0-187.0	161.3	140.0-180.0	63.0, 62.0	41.0, 57.0	.0	.0	.0

* 30 sec. change. † Mean—6 experiments. ‡ Mean—3 experiments. § "Frozen" refers to 2 wk in deep freeze at −25° ± 1.0°C.
‖ EDTA demineralized bone. ¶ "C.S." refers to chondroitin sulfate.

The absence of metachromasia without Ca ion in the frozen bone may be an index of the inactive form of the molecule responsible for initiating calcification *in vitro.*

As seen in Table II, deep freeze storage *per se* does not destroy the enzymes of the glycolytic system. Such sections (Table I (a)) nevertheless lose their ability to calcify *in vitro.* Those sections removed from the animal and shaken with calcium chloride prior to freezing have lost practically all of their enzymes but nonetheless retain the ability to calcify *in vitro* (Table I(c)). As shown in Table I(b) frozen sections which do not calcify *in vitro* and which appear to have an intact enzyme complement can be induced to calcify if they are shaken in calcium chloride solution after freezing. Control specimens, Table I(j,k), which were not frozen show a 1.5 (++++) *in vitro* calcification while the calcium chloride treated control slices show 2 (++++).

There is little change in enzyme activity after deep freeze storage (Table II). Yet the frozen tissue has lost the ability to calcify *in vitro* (Table I(d)). Prior to deep freeze storage, degree of *in vitro* calcification was 2 (++++). That the system retains calcifiability is shown by the observation (Table I (e)) that when frozen tissue is shaken for 1 hour in calcium chloride solution the mean degree of calcification is 1.7 (+++).

Most of the glycolytic enzymes were either destroyed or removed where bones were treated with $CaCl_2$ (Table II) or an equivalent amount of NaCl. However, a marked difference in the degree of *in vitro* calcification of these slices is apparent from data in Table I(c,h). In addition, the data indicate that calcium chloride was able to reactivate the sodium chloride inactivated slices. For example, the sections that were shaken in NaCl gave zero calcifications either before or after deep freeze treatment (Table I(g,h)). However, similarly treated sections when shaken for 1 hour in calcium chloride gave 1.3 (++++) and 1 (++++) *in vitro* calcifications (Table I(l,i)).

Table II shows the effect of calcium chloride shaking on enzyme activity of fresh carti-

TABLE III. Influence of Heating at 65°C on Calcification *In Vitro*.

Treatment	Degree of calcification *in vitro* ($Ca \times P = 60$) Mean
Tibial slices heated 30 min. at 65°C	0 (0)
" " " 60 " " "	0 (0)
" " " 30 " ; shaken 1 hr in 150 meq/l of $CaCl_2$	1.0 (++)
" " " " " ; " " " 300 " " "	2.0 (+++)
" " " 60 " ; " " " 150 " " "	0 (0)
" " " " " ; " " " 300 " " "	1.7 (++)
Fresh, unheated slices	1.5 (++++)

lage. It is clear that most of the enzyme activity is lost by shaking in calcium chloride, yet ability to calcify *in vitro* is retained(7).

In Table III it may be seen that heating tibial slices in distilled water at 65°C for 30 or 60 minutes inactivates the calcifying mechanism of the preosseous cartilage. Robison *et al.*(2) had observed complete inactivation in 10 minutes at 60°C. However, this inactivation could be reversed to some extent with 150 milliequivalents of Ca^{++}/l or to a greater extent with 300 milliequivalents of Ca^{++}/l, and though the resulting *in vitro* calcification was not as marked as in the controls, it had the typical honeycombed structure precisely situated in the matrix of the hypertrophic epiphyseal cartilage. This reconfirmed the earlier work of Sobel and Hanok(7) on the reversible inactivation of calcification *in vitro*.

The effect of heating at 65° for 30 minutes on the enzymes of the cartilage tissue is shown in Table II along with data from fresh, untreated controls. It is obvious that heating even for 30 minutes destroys all traces of enzyme activity. Yet it is apparent from the data shown in Table II that such slices after shaking in calcium chloride still have some ability to calcify *in vitro*.

From Table IV it is apparent that EDTA treatment alone (portion "A") inactivated the calcifying mechanism. However, after $CaCl_2$ treatment (portion "B") a grayish, silver deposit was observed in the provisional zone of the epiphyseal cartilage which, though atypical, was situated in the calcifiable provisional zone of the tissue. Some stained areas in the epiphyseal trabeculae, in the metaphyseal osteoid and traces in the diaphysis were observed. The chondroitin sulfate treated slices (portion "C") showed inactivation while the $CaCl_2$ treated counterpart (portion "D") showed good reactivation with typical, honeycombed structure in the epiphyseal cartilage and positive stains in the osteoid, diaphysis and epiphyseal trabeculae. The enzyme studies, on the other hand, showed no activity in either the "A" or "D" portions.

Of interest is the recent *in vivo* experiment in which EDTA decalcified cortical bone of rats was broken down in a Waring blendor

TABLE IV. Influence of Chondroitin Sulfate and Calcium Chloride on Calcification *In Vitro* of Hypertrophic Epiphyseal Cartilage of EDTA Demineralized Tibial Slices.

Portion	Preliminary treatment	Degree of calcification *in vitro* ($Ca \times P = 60$) Mean
"A"	20 hr in basal salt solution	0 (0)
"B"	*Idem*; shaken 1 hr in 500 meq/l $CaCl_2$	1.0 (+++)*
"C"	18 hr in 1% chondroitin sulfate; 20 hr in basal salt solution	0 (0)
"D"	*Idem*; shaken 1 hr in 500 meq/l of $CaCl_2$	2.5 (+++)†
	Fresh untreated controls	2.0 (++++)

* Grayish, atypical silver stain.

† Dense, dark gray silver stain; positive stains in diaphysis, epiphyseal trabeculae and metaphyseal osteoid.

and implanted in a trephined hole in the skull of a rat. A portion of the "blendorized" material was treated with chondroitin sulfate and then implanted in another trephined hole in the skull. After 6 weeks, the hole that received the chondroitin sulfate treated material showed excellent healing of the trephined area as compared to the decalcified, non-chondroitin sulfate treated material. Gross and microscopic examination showed no foreign body or other deleterious effects. X-ray shadow photographs confirmed the gross findings.

These experiments confirm the previous observations(8,10) that led to the postulate that a special structure of collagen which can be produced by chondroitin sulfate may be the essential part of the calcifying mechanism.

The results of EDTA treatment on the glycolytic enzymes in the cartilage and in the shafts and epiphyseal trabeculae are summarized in Table II. It is clear that treatment with EDTA and chondroitin sulfate has led to the destruction of all enzyme activity in both types of tissue under investigation. Nevertheless, calcifiability *in vitro* is retained.

Although the precise roles of phosphatase and the phosphorylative enzymes of the glycolytic cycle are still obscure, there is some histochemical evidence that, *in vivo,* they may be related in some way to preosseous cellular metabolism(11) and to the elaboration of the components of the matrix(12,13) that is calcifiable and that will, indeed, calcify provided sufficient amounts of calcium and phosphate ions are available in the extracellular fluids that bathe the matrix. It is also possible that the glycolytic cycle, in producing ATP, activates the mechanism responsible for the initiation of calcification(14).

From the present study it appears that, at least those enzymes investigated and therefore the glycolytic system as a whole, are not essential for *in vitro* calcification but that calcium ions are required to activate, reactivate or preserve the calcifying mechanism, which resides in collagen of special structure or the collagen-mucopolysaccharide moiety in the preosseous cartilage and in such configuration as to receive and react with the mineralizing ions to produce the bone salt, apatite. Several reports have appeared that are in harmony with this concept(8,15-22).

Summary. (1) Glycolytic enzymes and their relation to *in vitro* calcification of the preosseous cartilage of rachitic rat bone tissue have been studied. The findings indicate that although glycolytic enzymes are present in deep frozen tissue, ability to calcify *in vitro* is lost. However, if the tissue is treated first with calcium chloride and then stored in the deep freeze, the glycolytic enzymes are either absent or present only in trace amounts while the calcifying mechanism remains virtually intact. (2) Bone sections heated at 65°C and then treated with calcium chloride retain their ability to calcify *in vitro* despite the destruction of the glycolytic enzymes during the heating process. (3) Demineralization of bones results in a loss of glycolytic enzyme activity. However, when the demineralized bones are treated with chondroitin sulfate and then with calcium chloride, the calcifying mechanism is restored but not the glycolytic enzyme activity. (4) It is concluded that glycolytic enzymes are not part of the minimal system required for calcification.

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Stimulation of Vit. B_{12} Uptake in Tissue Slices by Intrinsic Factor Concentrate.[*] (23388)

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For several years we have held the view that intrinsic factor may influence absorption of vit. B_{12} by tissues other than the intestinal mucosa. Currently it is held that intrinsic factor is a mucopolypeptide of about 5,000 molecular weight(1). This size would not preclude the possibility of its being absorbed into the blood stream and hence becoming available for possible action on all tissues. However, no reports have yet appeared that demonstrate the effect of intrinsic factor on peripheral tissues.

Data are presented that demonstrate the stimulatory action of intrinsic factor on uptake of vit. B_{12} by slices of rat liver[†] or kidney *in vitro*. It appears that these observations may form the basis for an *in vitro* assay of intrinsic factor.

Materials and methods. Wistar strain, weanling rats were fed a diet of the following percentage composition: vitamin-free casein, 22; sucrose, 68.5; salts mixture (U.S.P. No. 2), 4.15; crisco, 4; iodinated casein, 0.04 or 0.05; L-cystine, 0.2; choline, 0.1. Each 100 g of diet contained α-tocopherol, 2.5 mg; menadione, 50 μg; vit. A. 75 I.U.; vit. D, 25 I.U.; riboflavin, 600 μg; thiamin, 400 μg; pyridoxine hydrochloride, 400 μg; calcium pantothenate, 1600 μg; niacin amide, 2000 μg; paraaminobenzoic acid, 10 mg; inositol, 20 mg; biotin, 10 μg; and folic acid 20 μg. In diets *B* and *D* vit. B_{12}, 2 μg/100 g diet was added. Iodinated casein was not added to diets *C* and *D*, (Table II). After 4 weeks on the diets the animals in groups A and C showed depressed growth rates and animals from all groups were ready for experiments. Rats were sacrificed by decapitation and the liver quickly removed and cooled on cracked ice. Slices were prepared 0.5 mm thick, blotted on filter paper, weighed on torsion balance before being placed in 7 ml Warburg flasks, and incubated at 37°C. Each flask contained a total volume of 3 ml of buffer pH 7.4 described by Hastings *et al.*(2). The radioactive vit. B_{12}, intrinsic factor concentrate[‡] and other additives were made up in buffer solution. The system was equilibrated with gas (5% CO_2, 95% O_2) and vit. B_{12} tipped-in from the side arm after temperature equilibrium had been reached. After incubation, the slices were removed from the flask,

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[†] Preliminary report, *Fed. Proc.*, 1957, v16, 393.

[‡] Intrinsic factor concentrate was prepared from hog mucosa and was supplied by Abbott Laboratories, North Chicago, Ill. This preparation had a biological oral dose potency of about 5 mg/day.

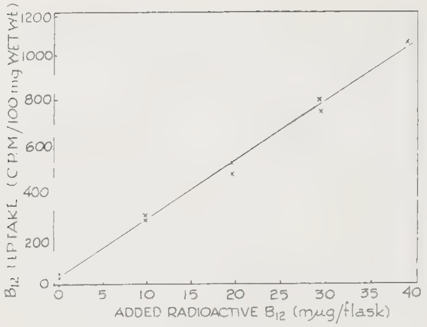

FIG. 1. Uptake of vit. B_{12} by rat liver slices plotted against varying amounts of radioactive vit. B_{12} added to each flask. No intrinsic factor concentrate added.

washed 10 sec. in 5 ml of buffer, and placed in cuvettes for assay in center-well scintillation counter. Cobalt^{60} labeled activity vit. B_{12}§, μc/μg, was diluted to 0.05 μc/ml. Potassium pyruvate was prepared(3) and added to the incubation mixture at a final concentration of 60 mM/l. The data are reported as counts/minute (C.P.M.)/100 mg tissue wet weight, corrected for background. Livers were analyzed for vit. B_{12} content using the *L. leishmannii* ATCC 7380 as the test organism. Homogenates were prepared and digested with pancreatin for 24 hours at 37°C. The values are reported in mμg/g wet weight of liver and have been corrected for non-vit. B_{12}-like materials by reporting only the alkali-labile values.

Results. Fig. 1 shows the results of an experiment in which the effect of vit. B_{12} concentration on vit. B_{12} uptake by liver slices is demonstrated. Remarkably good agreement is obtained between duplicate determinations, and uptake is proportional to concentration of vitamin over the range tested during a 4 hour incubation.

The effect of washing tissue slices after incubation, upon uptake of vit. B_{12} was determined. (Fig. 2). Slices of liver were incubated 4 hours, removed from the flask and washed 10, 60 or 180 seconds in buffer. Washing control slices for these intervals reduced uptake of B_{12} only slightly, whereas slices to which 100 μg of intrinsic factor concentrate had been added lost a significant amount during the first 60 seconds. However, a significant stimulation in uptake remained even after the longest wash period. Wash periods of 1 or 3 minutes seemed to reduce the net stimulation due to added intrinsic factor concentrate and for this reason a 10 sec. wash period was selected for routine use. Incubation of slices in medium with radioactive B_{12} for 10 sec. followed by washing for 10 sec. exhibited minimal uptake, 48 ± 3 C.P.M/100 mg. Furthermore, it was found that incubating slices in medium without radioactive B_{12} for 4 hours prior to tipping in radioactive vitamin, did not increase amount absorbed by the slice in 10 seconds.

The influence of time on uptake of vit. B_{12} is demonstrated in Fig. 3. Uptake by control slices essentially reached maximum after 1 hr. of incubation. However, when 100 μg of intrinsic factor concentrate was added, the uptake was more than twice that of control slices after 4 hours.

Stimulation of vit. B_{12} uptake appears to be a function of the amount of intrinsic factor concentrate added in Fig. 4. As little as 5 μg and as much as 100 μg appear to stimulate

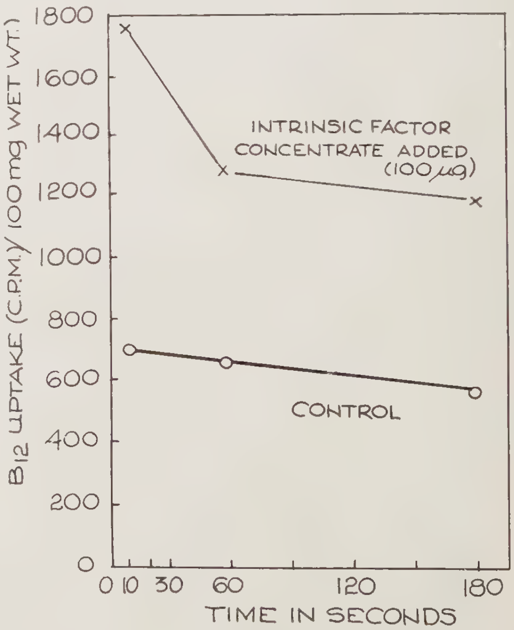

FIG. 2. Uptake of vit. B_{12} by rat liver slices plotted against length of time taken to wash the slice free of adsorbed radioactive vit. B_{12}.

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§ Cobalt^{60} labeled vit. B_{12} was furnished by Merck and Co., Rahway, N. J.

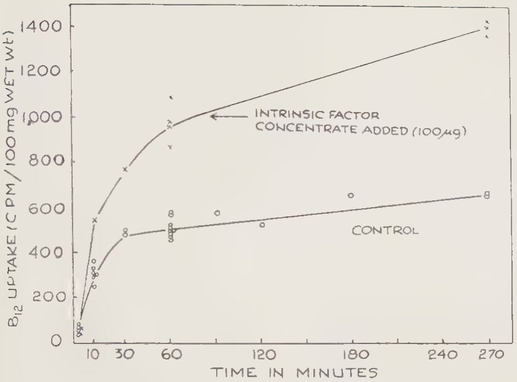

FIG. 3. Uptake of vit. B_{12} by rat liver slices plotted against length of incubation. Stimulatory effect of intrinsic factor concentrate is demonstrated.

the uptake of vit. B_{12}. Increased amounts (500 to 1000 µg) of this preparation caused inhibition in uptake, presumably because the concentrate bound the radioactive vit. B_{12}, thus preventing its uptake by the slice.

To determine whether or not stimulation of vit. B_{12} uptake of liver slices is related to the intrinsic factor content of the concentrate, the experiments summarized in Table I were done. The data are not conclusive but they do suggest that stimulation of B_{12} uptake is a function of intrinsic factor. For example, preheating intrinsic factor concentrate in boiling water for 30 minutes negates the stimulation. Pink-protein,‖ a cobalamin protein complex which has no intrinsic factor activity (4), has no stimulatory effect. Crystalline pepsin and lysozyme had no effect although crude gelatin may have had a slight effect.

TABLE I. Specificity of Intrinsic Factor Concentrate for Stimulating Uptake of Radioactive Vit. B_{12} by Rat Liver Slices.

Additions to flask*	No. of exp.†	C.P.M. per 100 mg	% of control
None	20	870	100
Intrinsic factor concentrate	16	1395	160
Intrinsic factor concentrate, heated	10	1065	113
"Pink protein"	6	960	105
Crystalline pepsin	6	1096	103
Lysozyme	6	1154	108
Crude gelatin	4	1277	119

* Each addition was made 100 µg/flask.
† 4 hr incubations.

For most experiments, slices were prepared from livers of rats reared on a diet devoid of added vit. B_{12} and including 0.04 or 0.05% iodinated casein, diet *A*. However, a study was made to determine whether or not it was necessary to use hyperthyroid-vit. B_{12}-deficient rats. In Table II data are shown which compare liver slices from rats given 4 different diets. Intrinsic factor concentrate will stimulate uptake of vit. B_{12} by liver slices from rats receiving any one of the 4 diets, but uptake in the absence of intrinsic factor concentrate is less in normal animals than hyperthyroid rats.

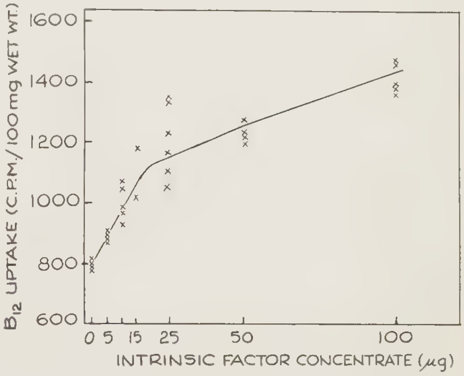

FIG. 4. Uptake of vit. B_{12} by rat liver slices plotted against amount of intrinsic factor concentrate added to each flask.

Uptake of vit. B_{12} by kidney slices is also stimulated by intrinsic factor concentrates. Control slices absorbed 562 ± 43 C.P.M./100 mg but in the presence of 100 µg intrinsic factor concentrate 789 ± 86 C.P.M./100 mg was absorbed. The stimulation was statistically significant at the 5% level.

Discussion. Data presented demonstrate that intrinsic factor concentrates will stimulate the uptake of vit. B_{12} by slices of liver and kidney. The data indicate that this stimulation is a function of intrinsic factor in the concentrates used inasmuch as pink-cobalamin-protein, that stimulated the combining of vit. B_{12} on serum proteins(5), had no effect in this system. Furthermore, concentrates that were preheated in boiling water for 30 minutes lost their stimulatory action. Lysozyme, which has been shown previously(6)

‖ "Pink-protein" was supplied by Dr. K. W. Thompson of Organon Inc., Orange, N. J.

TABLE II. Effect of Diet on Vit. B_{12} Uptake by Slices of Rat Liver.

				C.P.M./100 mg of liver	
Diet	Iodinated casein	Vit. B_{12}	No. of exp.*	Control	Intrinsic factor concentrate added, 100 μg
A	+	—	6	957 ± 71†	1397 ± 71
B	+	+	6	953 ± 83	1535 ± 29
C	—	—	10	626 ± 34	1249 ± 93
D	—	+	10	620 ± 21	1337 ± 82

* 4 hr incubations. † Stand. error of mean.

to bind B_{12} and make it unavailable to microorganisms, is without intrinsic factor activity by bioassay and in the system described above. This suggests that the binding of vit. B_{12} *per se* may not be the only mechanism involved whereby intrinsic factor stimulates the uptake of vit. B_{12} by liver slices. In some instances binding of vit. B_{12} may prevent stimulation owing to competition between the material and the slice of tissue.

It was anticipated that vit. B_{12}-deficient rat liver slices would be necessary to show an effect of intrinsic factor on uptake of vit. B_{12}. Actually, stimulation of B_{12} uptake was observed in slices from rats given each of the 4 diets tested; the only difference being in the uptake of vitamin by control slices. Hyperthyroidism seems to cause increased uptake of radio-B_{12} quite unrelated to the presence or absence of vit. B_{12} in the diet, or the level of vit. B_{12} in the liver. Inasmuch as the vit. B_{12} level in the liver was depressed from 87 ± 9 (diet B; B_{12} supplemented) to 30 ± 15 mμg/g in animals given a diet to which no B_{12} was added (diet A), it seems that the effect noted was probably unrelated to the level of vit. B_{12} in the liver.

The technic described is capable of detecting small amounts of intrinsic factor concentrate and further refinements and experience may make it the most sensitive assay for intrinsic factor thus far reported in the literature. The development of a specific and sensitive *in vitro* method for determination of intrinsic factor may aid in its isolation, identification, and also in elucidating its physiological action.

Recently we have demonstrated in our laboratory that homogenates of liver will combine vit. B_{12} and this can be stimulated by addition of intrinsic factor concentrate. When these homogenates are centrifuged at 25,000 x g, stimulation of vit. B_{12}-combining was observed only in the supernatant fraction and none in the particulate fraction which sedimented under these conditions. Full details of these experiments will be published subsequently. These data are interpreted to indicate that intrinsic factor is absorbed into the cells of the liver slice and thus exerts its stimulatory action intracellularly rather than exerting a non-specific membrane adsorption phenomenon.

It has been demonstrated previously that intrinsic factor concentrates and gastric juice will stimulate vit. B_{12}-combining on certain of the plasma proteins(7). For this action of intrinsic factor to be of physiological importance it was postulated(5) that intrinsic factor is absorbed into the blood stream where it acts not only to conserve the dietary vit. B_{12} by combining it with serum proteins but also facilitates the absorption of vit. B_{12} by peripheral tissues in the same manner as it facilitates absorption of vit. B_{12} from the gut. Evidence is presented here in support of this hypothesis.

Summary. It was demonstrated that intrinsic factor concentrate stimulates the uptake of radioactive vit. B_{12} in slices of rat liver and kidney, *in vitro*. Evidence presented indicates the specificity of this stimulatory action. The stimulation is a function of concentration of intrinsic factor concentrate added, and may therefore be the basis of an *in vitro* assay for intrinsic factor. It was not necessary to use vit. B_{12} deficient tissues to show the stimulation effect and it was observed that hyperthyroidism caused increased uptake of vit. B_{12} in the absence of added intrinsic factor concentrate. A brief discussion of the possible function of intrinsic factor was

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Plasma Protein Changes in Parakeets with Pituitary Tumors.[*] (23389)

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Among the commonest tumors of the parakeet *Melopsittacus undulatus*, are adenomas and carcinomas of the pituitary(1). Over 160 cases have been examined in this laboratory, and in the past 4 years one of the tumors has been carried through 14 passages by subcutaneous transplantation(2). Both primary tumors and transplants often produce marked obesity and hyperglycemia. Bioassays of the transplanted tumors suggest the presence of somatotropin, but they have been negative for other pituitary hormones(2). The plasma proteins show a striking quantitative change in the electrophoretic pattern (Fig. 1), and a qualitative difference from the normal after ultracentrifugation.

Materials and methods. Tumors to be transplanted were minced in 0.9% NaCl and injected beneath the skin of the breast. Cortisone, total body x-irradiation, or other form of pretreatment of recipient birds was never used. Total plasma protein and blood sugar were determined by a microphotometric application of the Folin-Ciocalteu procedure(3) and by the Folin-Wu technic(4) respectively. Parakeets with primary pituitary tumors were exsanguinated by cardiac puncture. Clotting was prevented with heparin in most instances; in several cases no heparin was used and the electrophoretic pattern of the serum so obtained compared with that of plasma. The method of paper electrophoresis did not demonstrate a significant difference between serum and plasma. No separate peak corresponding to fibrinogen could be detected in the plasma, a finding similar to that observed in mice(5) and fowls(6). Birds with transplanted pituitary tumors were also exsanguinated, particularly if blood sugar determinations and ultracentrifugation were to be carried out on the same sample. When serial protein determinations were made on a single bird, blood was collected in heparinized capillary tubes from a wing vein. Plasma was applied in amounts of 10 cu mm to Whatman 3-mm filter paper that measured 10 x 14 cm, providing space for simultaneous testing of several samples. Using a conventional vertical principle electrophoretic cell designed by one of us (R.W.), Veronal buffer at pH 8.6, u 0.05 with 5% glycerine, a current of 150-170 volts delivering 15-20 milliamperes was applied to the paper for 4 hours. After heat fixation the papers were stained with Bromphenol blue for protein and Sudan black B for lipoprotein. The papers were then cut into strips each bearing the pattern of a single sample, and scanned with a Spinco Analytrol that permitted simultaneous area integration. Pooled human serum served as a control and mobility reference. The protein fractions in parakeet sera were not isolated, therefore their dye-binding capacities could not be de-

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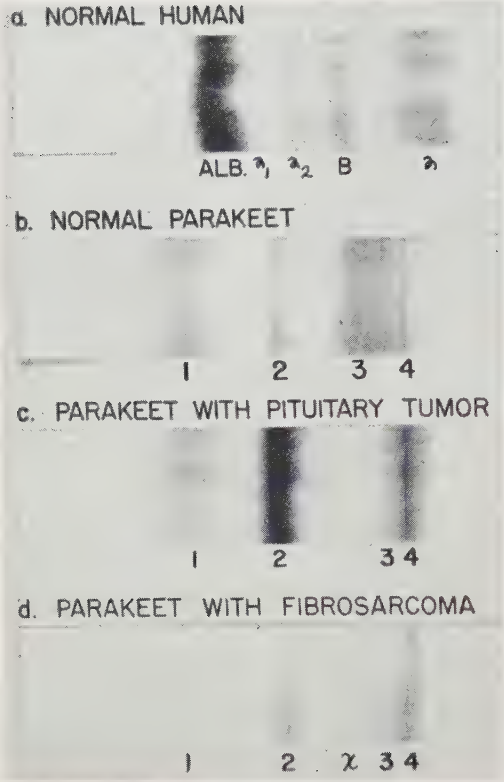

FIG. 1. Representative electrophoretic patterns of parakeet plasma with a human serum pattern as mobility reference.

termined directly. They were assumed to be similar to those of the identically treated human sera. This assumption is supported by the results of boundary electrophoresis, which were similar to those obtained by paper electrophoresis. An Aminco-Stern boundary electrophoretic apparatus was used to determine the electrophoretic mobilities of plasma protein components with Veronal buffer, pH 8.6, u 0.1. To supply the relatively large volume of plasma needed for these studies it was necessary to use pooled samples from several birds. Mobilities were calculated from the ascending boundaries and are reported in Tiselius units. Analysis by ultracentrifugation was performed on a Spinco Model E optical ultracentrifuge at 59,780 r.p.m. Plasma from individual birds as well as samples of pooled plasma from several birds were used. Sedimentation constants were calculated from 3 or more dilutions of each sample and are reported in Svedberg units.

Results. Normal Parakeets. In 15 normal birds the average total plasma protein was 3.34 g %, range 1.98 to 4.74 g %. The plasma from each of 29 normal parakeets and the pooled plasma of 40 normal birds was subjected to zone and boundary electrophoresis. The birds ranged in age from 2 months to 2 years, the sexes were equally distributed. Four major components are recognizable(7), on the basis of their mobilities they are similar to those found in other birds(8,9) and man. Component 1 has a mobility of 8.5, and may be albumin. The components 2, 3, and 4 have mobilities of 4.2, 3.25, and 2.25 respectively and are probably globulins; however, they have not yet been identified by chemical methods. There is considerable individual variation in the quantity of each of these 4 fractions present in the plasma (Fig. 2). The average percentile of component 1 is 32 (range 17-36%), of 2 is 16 (6-28%), of 3 is 22 (8-45%), and of 4 is 29 (17-48%). An affinity for the lipid stain Sudan black B was pronounced in component 1, less intense in 3 and 4 which stained as a unit, and barely perceptible in component 2.

Birds with primary pituitary tumors. In 9 parakeets a diagnosis of pituitary tumor could be made before death. The birds were exsanguinated by cardiac puncture and the presence of a pituitary tumor confirmed at autopsy (Table I). The plasma electrophoretic pattern was characterized by a striking increase in component 2 (Fig. 3) with an average percentile of 57 (range 34-67%) as compared with a normal of 16 (range 6-28%). The other components were correspondingly decreased, particularly component 1 which fell to 10% (range 3-16); component 3 averaged 14% (range 5-34), and component 4 was 21% (3-35). Several of the birds were

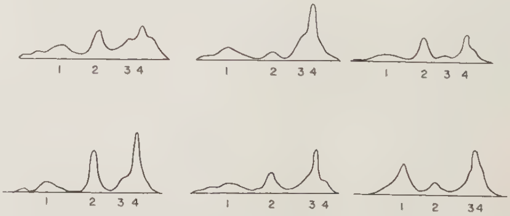

FIG. 2. Plasma protein patterns of 6 normal parakeets showing usual quantitative variation in the 4 components.

TABLE I. Birds with Primary Pituitary Tumors.

Date	Sex	Age (yr)	% tot. prot. as component 2*	Remarks
3/ 3/55	♂	4	56	Fatty and glycogen-rich liver, blood sugar 366 mg %
18	♀		50	5 mm tumor, blood sugar 97 mg %
4/13	♂	1	64	Tumor infiltrates 4th ventricle, fatty liver
10/13	♀		53.5	Large tumor invading retro-orbital tissue
3/16/56	♂		57.4	2 mm tumor, glycogen-rich liver
12/ 6	♀	2	67	Large tumor invading base of skull
1/29/57	♀	2	61	4 mm invasive tumor, glycogen-rich liver
2/ 6	♂	4	66	5 mm infiltrating tumor, fatty liver
3/ 7	♂	3	34	Large tumor invading skull and retro-orbital tissue

* In 29 normal parakeets avg value of component 2 was 16% (range 6-28%).

obese, in one the blood sugar level was elevated to 366 mg%; the normal is 150-250 mg%. Except for frequency of fatty and glycogen-rich liver, no consistent changes were observed in gross or microscopic appearance of the viscera or other endocrine glands.

Birds with transplanted pituitary tumors. On June 18, 1953 a massive pituitary tumor was found invading the roof of the mouth and retro-orbital tissue of a male parakeet. The tumor grow well when transplanted subcutaneously in other parakeets and since then has been carried through 14 serial passages. Electrophoretic studies were made on 23 birds bearing the tumor in its 3rd to 9th, and 12th passages (Table II). At the time of bleeding all but 5 transplants were over 100 days old; the average was 127 days with a range of 38-240 days. Only one tumor was less than 1 cm in diameter; the average was 2.1 cm with a range of 0.5 to 4.5 cm. Blood sugar levels were obtained in 13 instances on the same blood sample that was used for electrophoresis. Three were high normal; *i.e.*, 171, 212, 250 mg%, the remaining 9 were elevated ranging from 324 to 1768 mg% with an average of 887 mg%. The changes produced in the plasma electrophoretic pattern were identical to those observed in plasma of birds with a primary pituitary tumor; *viz.*, a pronounced increase in component 2 (Fig. 3). The average for component 2 in these birds was 51.5% of the total protein, with a range of 33-70%. This increase was very consistent and not altered by the number of passages, size of tumor, its duration of growth, or the blood sugar level. Nor was the increase of component 2 reflected in the total protein which when determined in 9 birds with a tumor in the 6th or 8th passage ranged from 2.64 to 5.93 g %. The average of 4.23 g % was slightly lower than the normal average of 4.74 g %.

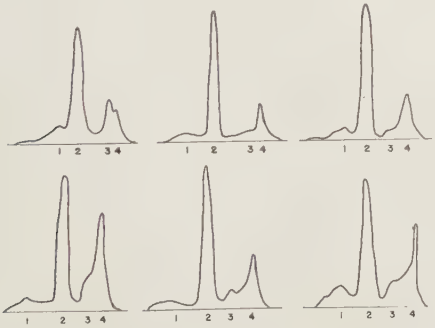

FIG. 3. Plasma protein patterns of 6 parakeets with pituitary tumors showing characteristic increase of component 2. Top row, birds with primary pituitary tumors; bottom row, birds with transplanted pituitary tumors.

To determine the time when the protein changes first become apparent, parakeets were inoculated subcutaneously with tumor from the 7th or 8th passages. One male and one female were then exsanguinated on each day for the first 7 days and then at weekly intervals for 14 weeks. Plasma electrophoresis first showed a definite increase of component 2 on the third day after transplantation, by the sixth day 45 to 65% of the total protein was present as component 2, a level 4 times that of the average normal. At this time the transplants were still very small and there was no gross evidence of growth. Blood sugar determinations made on the same blood sample as that used for electrophoresis, showed

TABLE II. Birds with Transplanted Pituitary Tumors.

Passage	Sex	Age of transplant (days)	Size of transplant (cm)	Blood sugar (mg %)	% tot. prot. as component 2
2	♀	180	1.5	1408	47
2	♂	240	1.0	1076	58
3	♀	137	3.5	1768	45
3	♀	150	2.0	504	60
3	♀	180	1.5	1332	60
3	♂	212	3.5	171	65
4	♂	78	1.5	212	59
4	♂	95	2.0	708	64
4	♀	125	1.5	250	35
4	♀	175	3.0	468	45
5	♀	115	2.0		55
5	♀	122	3.0	759	59
6	♂	80	1.5		56
6	♂	86	1.0		70
6	♂	93	1.5		47
7	♂	66	2.0		45
7	♂	185	3.0		41
8	♂	38	.5		33
9	♀	137	3.0		44
9	♂	137	2.0		55
12	♀	106	3.0	324	51
12	♂	106	4.5	352	44

considerable variability, in contrast to the constancy of the plasma protein changes. The first elevated blood sugar (298 mg%) was found in a male bird 9 weeks after transplantation when the tumor measured 8 mm in diameter. In one female with a 1.5 mm transplant, the blood sugar was 789 mg% by the tenth week and in a male with a 2 cm tumor transplant at 12 weeks, the blood sugar was 1220 mg%. However, at this time some birds with tumors of equal size still had blood sugar levels in the normal range.

Changes in plasma proteins following excision of the tumor were determined in 12 parakeets bearing 8-week-old transplants. In all birds component 2 of the plasma was elevated at the time of operation, 2 weeks later it had fallen to normal in all but 2 birds. In these, removal of the transplant had been incomplete as was indicated by a recurrence of the tumor at the original site within the following 2 months. During this time the plasma electrophoretic pattern of the other 10 birds remained normal.

Birds with other primary tumors. Electrophoresis was carried out on plasma of 5 birds with primary spontaneous tumors in which the pituitary was normal. Histologically 4 were fibrosarcomas, one was a rhabdomyosarcoma. In none did the electrophoretic pattern show an abnormal increase in component 2; instead, there was a decrease of all fractions associated with a marked hypoproteinemia (Table III, Fig. 1).

Birds with other transplanted tumors. In this laboratory 3 parakeet fibrosarcomas are being carried as subcutaneous transplants (10). The tumors originated in the retroperitoneal, subcutaneous, and retro-orbital tissues respectively. Electrophoretic patterns were obtained on the plasma of 16 birds bearing transplants of these tumors that were in the second to twelfth passage and measured 1.5 to 3.5 cm in diameter. The percentile distribution of the 4 components was within the normal range except in one bird where component 2 was greatly increased. At autopsy this bird proved to have a primary pituitary tumor.

A transplantable methylcholanthrene-induced skin carcinoma of the parakeet(11) provided an epithelial tumor to compare with the pituitary neoplasm for its effect on the plasma proteins. Birds bearing these trans-

TABLE III. Birds with Other Primary Tumors.

		Tumor				
Sex	Age (yr)	Type	Size	Site	% tot. prot. as component 2	Remarks
♀	2	Fibrosarcoma	2.5 × 2.5	Wing	14	Grew on transplantation
♀	2	"	2 × 1.5	Thigh	5	Met. to lung, kidney, liver
♂	3	" Adenocarcinoma	2.5 × 2 1.5 × 1	Orbit Kidney	20	Met. of sarcoma to spleen and liver
♂	–	Fibrosarcoma	2.3 × 2	Thigh	30*	No growth of transplants
♀	–	Rhabdomyosarcoma	2 × 2	Neck	12	*Idem*

* Marked hypoproteinemia, extremely low values for all components.

planted skin carcinomas in the second and third passage were exsanguinated when the tumors had reached a diameter of 2 to 3.5 cm. The electrophoretic pattern of the plasma protein was in the normal range and did not show the increase in component 2 characteristic of pituitary tumors.

Ultracentrifugation of plasma proteins. Four ultracentrifuge analyses were made on 2 different collections of pooled plasma from normal parakeets. Three components were seen: 1) a high density fraction with sedimentation constant of 19.7 representing approximately 5-8% of total protein, 2) a slower component that comprised approximately 5% of the protein and had a constant of 6.2, 3) the remaining 85-90% of the plasma protein with a sedimentation constant of 3.6. Nine analyses by ultracentrifugation were made on 2 different collections of pooled plasma from parakeets bearing transplanted pituitary tumors. The samples were first examined by electrophoresis for the presence of the abnormal component 2, the latter was found to represent 52% of the total protein. With ultracentrifugation 4 fractions were identified: 1) a high density component with a sedimentation constant of 17.9 representing approximately 5% of the protein, 2) a slower fraction having a sedimentation constant of 6.5, comprising 3-5% of the protein, 3) a new component with a sedimentation constant of 5.2 that constituted about 35% of the protein, 4) a fraction with a constant of 4.0 that contained the remaining 50-55% of protein. A fifth component showing flotation characteristics at the specific gravity of the diluent suggestive of a lipoprotein was also present.

The third component found in the ultracentrifuged plasma of tumor bearing birds is not seen in the plasma of normal parakeets. It may be a protein that is present in normal plasma in such small amounts that it is not detected by this method, or it is a new protein related to the presence of the pituitary tumor transplants.

Discussion. On the basis of its mobility during electrophoresis, the plasma protein fraction that is increased in parakeets with pituitary tumors is tentatively identified as a globulin. In man, serum globulins are increased in a wide variety of conditions but there is no consistent change characteristic of neoplastic disease(12). However, in multiple myeloma very large amounts of beta or gamma globulin may be demonstrated by electrophoresis(13). Reports on serum proteins of tumor-bearing rats and mice indicate great variability. Recently Bernfield and Homburger(5) have described a substantial increase in plasma alpha globulin of mice during 12 days after implantation of Sarcoma 180; upon further growth of the tumor the alpha globulin again decreases. Studies by Potter *et al.*(14) have demonstrated an increase of gamma globulin in a transmissible neoplasm of mice that closely resembles human multiple myeloma. These investigators suggest that the abnormal protein is produced by the neoplastic cells, a view supported by direct quantitative relationship between size of tumor and the amount of abnormal serum component. In the parakeet, component 2 is already markedly increased by the 3rd to 4th day after transplantation of 2-3 cu mm of tumor and before there is any visible evidence of growth. Although component *2* often shows a 5-fold increase, no consistent change in total protein was found. Preliminary studies on saline extracts of the tumor have indicated the presence of component *2*, but in lower concentration than in the blood of the host birds.

It is possible that the protein is formed by a target organ, *e.g.* the reticulo-endothelial system, which is activated by a hormone produced by the pituitary tumor. A hormone, perhaps somatotropin(2), secreted by the pituitary tumor probably produces the hyperglycemia that is often observed to accompany changes in plasma protein. The role of a hormone in altering serum proteins was recently reported by Schjeide and Urist(15). They observed that in roosters 24 hours after intramuscular injection of estrone, there is a large increase in serum beta lipoproteins, nearly complete disappearance of alpha lipoproteins and the appearance of 2 new components—a phosphoprotein and a lipoprotein. These changes reach a maximum in 5 days. The time elapsing between injections of estrogen and the appearance of new serum protein components in the rooster is the same as that observed in the parakeet following transplantation of the pituitary tumor.

The chemical identity of component *2* in the electrophoretic pattern has not yet been determined. The finding of a fourth plasma fraction by ultracentrifugation, which is not present in normal plasma, suggests that most of the increase in component *2* may be due to the production of a qualitatively different protein.

Summary. The first recognized manifestation of a primary or transplanted pituitary tumor in the parakeet is a marked increase in a plasma protein component, tentatively identified as a globulin. Analysis by optical ultracentrifugation suggests that this protein is qualitatively different from any present in normal plasma. Several weeks after appearance of the abnormal protein there is often an associated hyperglycemia and obesity. It is suggested that all these changes express the response of target tissues to one or several hormones secreted by the tumor.

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Presence of Plasma Erythropoietin in Hypoxic Rats with or without Kidney(s) and/or Spleen. (23390)

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The presence of an erythropoietic factor in the plasma of animals subjected to hypoxic hypoxia has been reported recently by Stohlman(1) and Prentice and Mirand(1a). Additional data from our laboratory bear out a time-concentration relationship of plasma erythropoietin (EPF) in hypoxic hypoxic rats. Knowing of this time-concentration of EPF, studies were carried out to determine whether this time-concentration relationship of plasma EPF can be altered in absence of the kidney(s) and/or the spleen. Jacobson *et al.*(2) reported recently that bleeding and cobalt chloride administration did not produce circulating erythropoietin in the absence of the kidneys and postulated that the kidney is the site of production of EPF. The purpose of this report is to demonstrate that hypoxic hypoxic rats with or without their kidney(s) and/or spleen can produce erythropoietin.

Material and methods. Sprague-Dawley rats were placed in chambers constructed of plexiglass. Into each chamber was directed a mixture of nitrogen and oxygen, the proportions of which, along with ambient pressure, determined the simulated altitude. Flow of nitrogen and oxygen was regulated by a rotameter type of flow-meter and remained quite constant over the experimental period. The chamber O_2 concentration was checked by reading of the oxygen and nitrogen flowmeters and also by a portable Beckman Oximeter. Random air samples were also taken from the chamber in a Douglas bag and checked with a mass spectrograph for O_2, N_2 and CO_2. Good agreement in results was obtained. A flow of approximately 5 liters/minute was maintained through the chamber and soda lime and sulfuric acid were placed in the chamber to minimize CO_2 and humidity levels.

Sprague-Dawley rats, 2 to 3 months old, used as donors, were exposed in a chamber to an atmosphere of approximately 10% O_2 for the time intervals shown in Table I. Rats of another group, used as donors, were nephrectomized and/or splenectomized and caution was taken during the operation that no bleeding occurred. They were then immediately placed in an atmosphere of 10% O_2 at various time intervals (Table II). Food (Purina Laboratory chow) and H_2O were available to rats in the chamber at all times. At the end of the respective intervals the donor rats were withdrawn from the chamber and promptly exsanguinated from the dorsal aorta. A final hematocrit was done on each rat and no animal with hematocrit below 40 was used as a source of plasma. Plasma obtained was frozen and stored until used.

Recipient animals were 3-month-old Sprague-Dawley rats which were used as assay animals approximately 15-20 days after hypophysectomy(3,4). They received 2 cc of donor rat plasma to be tested I.V. via the jugular vein on 2 successive days. On day 3 approximately 1 μc Fe^{59} was given I.V. via femoral vein and on day 4, a 24-hour Fe^{59} uptake was done. The results for each group of animals were expressed as the average 24-hour Fe^{59} uptake for the particular group.

Results. The time-concentration relationship of plasma EPF in hypoxic hypoxic rats with kidneys and spleen intact is clear cut (Table I). The concentration is approxi-

TABLE I. Hypophysectomized Rats (Recipient) Receiving Plasma from Normal Rats (Donor) Kept in 10% Oxygen for Varying Periods of Time. Erythropoiesis in recipient as judged by Fe^{59} uptake.

Treatment of donor rats at 10% O_2 atmosphere at various intervals (hr)	No. of recipient rats	24 hr Fe^{59} uptake
4	4	15.6 ± 5.88*
8	4	32.0 ± 4.20
24	5	34.5 ± 7.7
48	5	2.6 ± 1.8
120	6	2.6 ± 1.4
0 (normal plasma)	4	2.7 ± 1.86
0 (untreated)	3	3.3 ± 1.85

* ± S.D.

TABLE II. Hypophysectomized Rats (Recipient) Receiving Plasma from Normal Sprague-Dawley Rats (Donor) Which Were Nephrectomized and Splenectomized and Kept in 10% Oxygen for Varying Periods of Time. Erythropoiesis in recipient as judged by Fe^{59} uptake.

Treatment of donor rats prior to placing in 10% O_2 atmosphere	Interval at 10% O_2	No. of recipient rats	24 hr Fe^{59} uptake
Unilateral nephrectomy	4	5	13.9 ± 4.13*
	8	5	31.2 ± 5.84
	24	6	40.0 ± 5.66
Bilateral nephrectomy	4	6	17.9 ± 3.06
	8	5	13.2 ± 4.23
	24	6	37.9 ± 2.53
Sham nephrectomy	4	6	21.8 ± 4.84
	8	5	29.1 ± 6.41
	24	6	35.4 ± 7.70
Splenectomy	4	3	25.1 ± 1.70
	8	5	33.3 ± 6.37
Splenectomy-unilateral nephrectomy	4	6	21.8 ± 5.37
	8	5	37.8 ±11.45
Splenectomy-bilateral nephrectomy	4	4	13.4 ± 5.36
	8	6	17.6 ± 1.91
Sham splenectomy-nephrectomy	4	6	22.4 ± 5.12
	8	6	35.1 ± 4.78
Normal rat plasma	—	5	7.6 ± 2.69
Untreated	—	6	6.7 ± 4.0

* ± S.D.

Assays in all groups were carried out at the same time and under similar circumstances. In no instance did similar treated rats at normal atmosphere show a significant elevation of erythropoietin titers.

mately 5 times the control level at 4 hours. At 8 hours the titer reaches 10 times control level and remains approximately the same at 24 hours. By 48 and 120 hours the titer has dropped back to control levels. The time-concentration relationship of plasma EPF in unilateral and bilateral nephrectomized animals subjected to hypoxic hypoxia is doubled at 4 hrs; doubled or quadrupled at 8 hrs, and 5-6 times greater at 24 hrs than the titers at control levels (Table II). Splenectomized, and splenectomized-unilaterally nephrectomized and splenectomized-bilaterally nephrectomized rats subjected to hypoxic hypoxia all show similar increases in EPF titers of about the same magnitude as that shown in the nephrectomized rats.

Nephrectomized and/or splenectomized rats exposed to normal atmosphere did not show any elevation of EPF. Their Fe^{59} values were comparable to values reported in Table II for hypophysectomized rats receiving normal plasma and those that were untreated.

Discussion. Increasing attention is being paid to the role of plasma erythropoietin in the control of erythropoiesis(6,7,8,9). The erythropoietin level in hypoxic hypoxia, a potent stimulus for the production of erythrocytes in an organism(5,6), is most relevant to the problem of control of erythropoiesis. Our studies indicate that EPF is elevated in the plasma of hypoxic rats during a limited time. This is in contrast to animals made anemic from bleeding or phenylhydrazine. Anemic rats seem to retain elevated levels of EPF in their plasma as long as they have a significant degree of anemia. The reason for this difference is not clear because present knowledge on relative rates of production and utilization or destruction of EPF in hypoxic hypoxia, or other erythropoietic stimulating states is not adequate.

Our studies also bear out that erythropoietin production can go on in the absence of the kidneys and/or spleen in rats subjected to hypoxic hypoxia. This is in contrast to Jacobson's *et al.*(2) and Erslev's(10) data which indicate that neither rats nor rabbits kept at a normal atmosphere after bilateral nephrectomy have the capacity to respond to a single dose of cobaltous chloride or a single massive hemorrhage by an elevation of plasma erythropoietin. Goldwasser *et al.*(11) recently reported that cobalt enhances red cell production by increasing formation of erythropoietin. Their preliminary report also emphasizes that the erythropoietin in anemic plasma has similarities to erythropoietin in cobalt plasma. It remains to be determined whether there are different erythropoietins in animals and human and whether there are different production sites and different release mechanisms for each. Assuming that there is just one kind of erythropoietin produced, our results then suggest that the kidney and spleen are not the production sites for erythropoietin. Gordon *et al.*(12) attempted, but unsuccessfully, to identify the site of production of circulating erythropoietin by assaying acidified boiled filtrates prepared of plasma, packed blood cells, and various organs. They postulate that the factor may actually be formed in plasma as a result of enzymatic action upon substrates provided by the blood-forming organs.

Summary. (1) Normal rats placed in a low oxygen atmosphere for time periods from 4 to 120 hours reveal definite elevation of plasma erythropoietin until 24 hours and after 48 hours erythropoietin drops back to normal levels as judged by Fe^{59} uptake assay using hypophysectomized rats. (2) On the basis of this time-concentration relationship of plasma erythropoietin in hypoxic hypoxic rats, no significant alteration of erythropoietin titers is evident in hypoxic rats with or without kidneys and/or spleen. (3) Hence, neither the kidney nor spleen is the production site of erythropoietin under hypoxic hypoxia.

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Cholesterol in Seromucoid Fraction of Normal and Pathologic Sera.*
(23391)

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Throughout this paper, seromucoid is defined as that fraction of human serum soluble in perchloric acid solutions and precipitable with phosphotungstic acid. Little is known of its properties and of its composition, which is probably heterogenous. It migrates in the greatest part with the α-globulin fraction of serum in the electrophoretic field(1,2). Its content of hexoses and hexosamine is high, while approximately 3.6%(3) to 4.2%‡ of its weight is constituted by materials soluble in hot alcohol, presumably lipids. Winzler *et al.* reported a negative Lieberman-Burchard's reaction in the lipid moiety isolated from mucoproteins of human serum(4). Rimington(5), on the other hand, using entirely different methods, identified cholesterol in a purified mucoprotein obtained from ox serum.

Our interest in the relationship of cholesterol to the seromucoid fraction was also stimulated by previous observations of significant alterations of seromucoid and of cholesterol in the serum of patients with lymphomas(6,7). Accordingly, we determined by chemical technics the cholesterol precipitated with the perchloric acid soluble, phosphotungstic acid insoluble fraction of serum in 6 normal subjects and in 21 patients all suffering from diseases in which significant multiple abnormalities of mucoproteins, seromucoid and cholesterol have been described (6 with Hodgkin's disease, 4 with reticulum cell sarcoma, 1 with lymphoblastoma; 5 with generalized carcinomatosis; 3 with acute leukemia and 2 with parenchymal liver disease).

Methods. Separation of seromucoid was carried out by a modification of the method of Winzler *et al.*(4), perchloric acid being used at a final concentration of 0.24 M instead of the standard 0.6 M(6). After filtration, the supernatant from the perchloric acid-treated serum (2 ml) was divided in 2 aliquots of 5 ml in glass-stoppered centrifuge tubes for determination of protein and of cholesterol. One ml of 5% solution of phosphotungstic acid in 2 N HCl was added to each test tube. After standing at room temperature for 10 minutes, all test tubes were centrifuged at 2,000 rpm for 10 minutes. Supernatant was discarded. Precipitate was washed once with 5 ml of phosphotungstic acid solution. Precipitate (seromucoid) was then analyzed for protein and for cholesterol. The biuret reaction, with casein as known standard(4), was used for determination of protein; a modification of Zlatkis' method(8) was used for determination of total serum cholesterol. This was as follows: after washing with phosphotungstic acid solution, the seromucoid precipitate was dissolved in 3 ml of aldehyde-free glacial acetic acid. Two ml of a solution of 1% ferric chloride in H_2SO_4 (1.84 s.g.) were added slowly. Tubes were shaken vigorously for 1 minute; 10 minutes later, when tubes had cooled off at room temperature, optical density of samples was read in Beckman spectrophotometer at 560 λ. Preliminary tests showed that phosphotungstic acid would not interfere with spectrophotometric reading for cholesterol at this wave length. Concentration of cholesterol was calculated by comparing the experimental readings with those of a curve showing the values obtained with solutions containing known amounts of cholesterol. A correction formula was used in the final calculations as indicated by Winzler *et al.*(4).

Results. 1. *Cholesterol in seromucoid fraction of normal serum* (Table I). The acetone-

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‡ Moschides, E., and Stefanini, M., unpublished.

TABLE I. Total Protein, Perchloric Acid Soluble Fraction (Seromucoid), Total Cholesterol and Cholesterol in the Seromucoid Fraction: Health and Disease.

					Seromucoid			
	Total serum protein, g %		Total serum cholesterol, mg %		Protein, mg %		Cholesterol, mg %	
	$\bar{X}$	σ	$\bar{X}$	σ	$\bar{X}$	σ	$\bar{X}$	σ
(a) Healthy individuals	7.18	.356	180.16	27.7	95.16	8.44	2.92	.22
(b) Hodgkins' disease	6.9	.62	164.2	31.4	164.2	49.2	6.1	1.035
(c) Reticulum-cell sarcoma, lymphosarcoma, lymphoblastoma	6.34	.567	159	30.8	189.54	18.46	6.12	1.9
(d) Acute leukemia	6.8	.51	232.3	16.6	122	15.1	2.4	.294
(e) Liver disease	6.1	.5	155	5	155	15	3.3	.2
(f) Metastatic carcinoma	5.84	.28	134	7	215.2	3.5	7.08	.74

$\bar{X}$ = Arithmetic mean. σ = Stand. deviation (s.d.).

alcohol extract of the seromucoid fraction of normal human serum exhibited positive Lieberman-Burchard's reaction. Untreated seromucoid fractions contained an average of 2.92 mg % cholesterol, as compared to an average total serum cholesterol value of 180.16 mg %. Free cholesterol and cholesterol esters were both found in the seromucoid fractions of normal and of pathological sera as well as in their acetone-alcohol extracts, in an average ratio of 1:4. Pathologic sera were more suitable for this type of study because of their higher seromucoid content. The following were average values in experiments on 3 such sera (metastatic carcinoma): (a) perchloric acid soluble protein (seromucoid), 200 mg %; (b) total cholesterol in this fraction, 14 mg %; (c) free cholesterol, 3.25 mg %. Thus, 81% of the cholesterol found in the seromucoid fraction was combined.

2. *Status of cholesterol in perchloric acid soluble fraction (seromucoid) of serum.* Normal and pathologic human sera were studied to establish the "chemical status" of cholesterol precipitated with the seromucoid fraction: (a) dilution of serum up to 1/10 with water and lowering of concentration of the perchloric acid reagent below a critical level of 0.225 M caused parallel and predictable reduction in yield of seromucoid and of cholesterol recovered from this fraction; (b) in a second set of experiments, however, cholesterol was extracted first by adding 5 ml of mixture of equal volumes of acetone and ethanol to 2 ml of both normal and pathologic serum. The seromucoid fraction was then precipitated and its cholesterol content determined. Only 40% of the expected cholesterol content could be found. Also, the seromucoid fraction was precipitated first from 2 ml of normal or pathologic serum and 5 ml of acetone-alcohol mixture were then added, to extract cholesterol. Again only 30 to 40% of the original cholesterol could be recovered in all instances from the washed seromucoid residue; (c) the precipitated seromucoid fraction contained only 15% of the expected cholesterol, after serum had been treated with 2.5 volumes of cold ethyl acetate; (d) cholesterol could be extracted entirely by treating the precipitated seromucoid fraction with 2.5 volumes of boiling alcohol; (e) in a final experiment, the seromucoid fraction was prepared from 50 ml of normal serum. After treatment of serum with perchloric acid, the supernatant (containing the seromucoid) was dialysed against concentrated dextrin. Fluid was thereby concentrated 6 times; pH was finally adjusted to 6.5. The solution, exhibiting a specific gravity of 1.10, was centrifuged for 22 hours in a Spinco Model L ultracentrifuge at 40,000 rpm (average 105,000 g).[§] Five fractions of equal volume were collected from top to bottom. The bottom fraction had a distinct yellow color. In addition, there was a well defined solid residue. Total seromucoid and cholesterol were determined in each fraction (Table II). Tendency of cholesterol to accumulate in the heaviest fraction was obvious.

The results of the various experiments excluded the existence of a true chemical bond-

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[§] By Dr. Peter Bernfeld, Cancer Research Unit, Tufts University Medical School.

TABLE II. Composition of Various Ultra-centrifugation Fractions from Seromucoid Fraction of Normal Human Serum (See Text).

	Protein, mg %	Cholesterol, mg %
Total seromucoid	98	2.5
Fraction 1 (top)	20	0
” 2	18	0
” 3	15	0
” 4	15	.5
” 5 (bottom)	15	1.3
Residue, mg	15	.7*

* Actual amount from 50 ml of normal serum.

age between mucoprotein and cholesterol.

3. *Cholesterol in seromucoid fraction of patients with lymphoma* (Table I). Total seromucoid was considerably increased in 9 of 11 cases, while total serum cholesterol was slightly decreased in this series. The cholesterol found in the seromucoid fraction, however, was significantly increased, in excess of the elevation that one would expect because of the larger content of seromucoid.

4. *Cholesterol in seromucoid fraction of serum of patients with miscellaneous diseases* (Table I). Sera of 5 patients with disseminated carcinomatosis showed a low total cholesterol content but uniformly high seromucoid content. Cholesterol found in the seromucoid fraction was also high. These findings were similar to those found in lymphoma, although, perhaps, of greater magnitude. Moderate elevation of the seromucoid fraction was observed in 3 patients with acute leukemia, who did not show anemia at the time of study. Serum was also obtained from 2 patients with parenchymal liver disease showing considerable increase of seromucoid[‖] and decrease in total cholesterol. Amount of cholesterol found in the seromucoid fraction, however, was within normal limits.

Discussion. It has been known for some time that cholesterol and some of its esters form complexes with the albumin and globulins of serum(10). Serum lipoproteins also are said to contain as much as 30% of cholesterol(11), although it has been postulated that cholesterol does not form a definite complex with lipoproteins(12). It would appear that no chemical bondage is involved in the case of the cholesterol found in the seromucoid fraction of serum by our technics. Rather, the finding is probably due to phenomena of co-precipitation, of adsorption, or of splitting of some cholesterol-containing fragments from other serum components by the action of perchloric acid and then co-precipitation with the seromucoid by phosphotungstic acid.

Although the distribution of cholesterol between protein and seromucoid fraction may, at times (Fig. 1), follow a "typical pattern,"

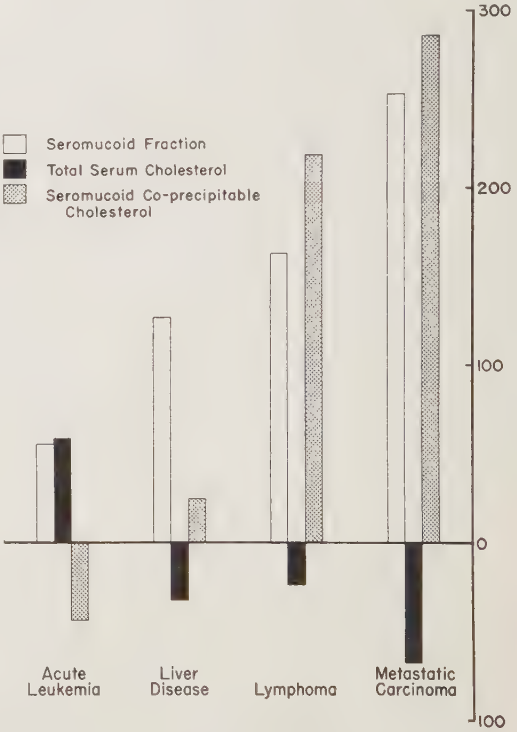

FIG. 1. Graphic illustration of changes in seromucoid, total cholesterol and cholesterol recovered from seromucoid fraction of serum of patients with various pathologic conditions. Horizontal line is avg normal value. Height of columns indicates % deviation from normal values. These data are those of single cases. Included is a selected case of liver disease, primarily to show the striking difference between this condition and disseminated malignancy. In both cases, seromucoid fraction is increased. Cholesterol recovered from this fraction, however, is low in liver disease, high in disseminated malignancy.

[‖] This is unusual(9). Cases were purposely selected to allow better comparison with findings in the serum of patients with lymphoma and metastatic carcinoma. In other cases of liver disease, when seromucoid is decreased, amount of cholesterol in this fraction is too low for accurate determinations.

these data are hardly of diagnostic value, since significant changes are found usually in patients with clinically obvious disease. Our study, however, by apparently showing the effects of perchloric acid upon cholesterol-containing components of serum, different from normal to pathologic conditions, may eventually bring a new approach in the investigation of disease states with abnormalities in cholesterol metabolism. Such possibility is being studied.

Summary. 1. A fairly fixed amount of free cholesterol and cholesterol esters is found in the perchloric acid soluble (seromucoid) fraction of human serum. Phenomena of co-precipitation or of adsorption are probably responsible for this finding. 2. Seromucoid fraction, total serum cholesterol, cholesterol found in the seromucoid fraction were studied quantitatively in pathologic sera obtained from patients with lymphoma, disseminated malignancies, acute leukemia and from selected patients with liver disease. Seromucoid fraction was elevated in all diseases. Cholesterol found in the seromucoid fraction was relatively increased in sera of patients with lymphomas and metastatic carcinomas; relatively or absolutely decreased in acute leukemias and liver disease. 3. The significance of these findings consisted in the indication of possible effects of perchloric acid on cholesterol-containing components of serum. Such effects seemed to vary from normal to pathologic conditions, thus suggesting the possible future value of this study in the investigation of abnormalities of cholesterol metabolism in disease.

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Pseudorabies Virus in Tissue Culture: Differentiation of Two Distinct Strains of Virus by Cytopathogenic Pattern Induced.* (23392)

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Scherer and Syverton(1) described the cultivation of pseudorabies virus in HeLa cells. They pointed out the rounding and granulation of the infected cells, and the presence of intranuclear inclusion bodies. The present paper describes the effects of pseudorabies virus on monkey kidney cells, and the separation of 2 strains of virus on the basis of their effects on the monkey kidney cells.

Materials and methods. Virus. The rabbit brain adapted Aujeszky strain of pseudorabies virus was supplied by the National Type Culture Collection. Before tissue culture adaptation, 18 egg passages had been made on the chorioallantoic membrane (CAM) of 13-day-old chick embryos. Each passage showed typical pocks. After adaptation to tissue culture, virus was recovered in the supernatant fluid after centrifuging infected cultures at 3,000 *rpm* for 5 minutes. This was used immediately for most experiments; if necessary it was stored at 4°C for intervals not exceeding 48 hours.

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‡ Material accepted as a thesis towards a degree of Master of Medical Science in Pediatrics in the Graduate School of Medicine, University of Pennsylvania.

Cell culture. The monkey kidney cell bottles were obtained from the Microbiological Associates, Inc., and maintained in a growth medium consisting of 2 parts calf serum, 98 parts of 5% lactalbumin hydrolysate in Hanks' balanced salt solution (BSS) with addition of penicillin and streptomycin in a concentration of 100 units, and 50 γ/ml, respectively. Cells were trypsinized by the addition of 20 ml of 0.5% trypsin (Difco 1:250) followed by incubation at 37°C for 20 minutes. After concentration at 900 *rpm* for 5 minutes, supernates were discarded, and sedimented cells were resuspended in fresh growth medium to yield a final concentration of 10^5 cells per ml. Aliquots of 20 ml of this cell suspension were transferred to bottles; 1 ml to screw cap tubes (Microbiological Associates, Inc.), and 5 ml to Petri dishes. Average yields of cells after 4 days at 37°C were in the range of 4×10^6 cells in bottle cultures, 3×10^5 in tubes, and 1×10^6 in the Petri dishes.

Virus assay. The $TCID_{50}$ (Tissue culture infecting dose) was determined in tube cultures inoculated with 0.1 ml of 10-fold dilutions of virus. The cytopathogenic effect was recorded daily for 5-7 days.

Antiserum. A swine pseudorabies virus antiserum was obtained through the courtesy of Dr. R. E. Shope. It showed no toxicity to the cell cultures.

Cytology. For detailed microscopy, cells were grown on coverslips inserted in the tubes prior to the seeding of the cells. When infected they were fixed by acetic Zenker solution, and stained with Hematoxylin-Eosin. Tube cultures were also stained with Giemsa *in situ.*

Experimental. When egg-adapted pseudorabies virus was passed serially in monkey kidney tissue cultures it was noted that different types of cytopathogenicity began to emerge. Originally the rounding and granulation of the cells, as described by Scherer and Syverton(1) were the only effects observed. However, after 3 bottle passages a few areas were noted in which small, multinucleated giant cells appeared. By a process of selection (to be described below) 2 strains of virus were separated; in one of these the predominant cytopathogenic effect was rounding and granulation of cells, and in the other giant cell formation and cytolysis. The cytologic characteristics of these 2 strains were as follows.

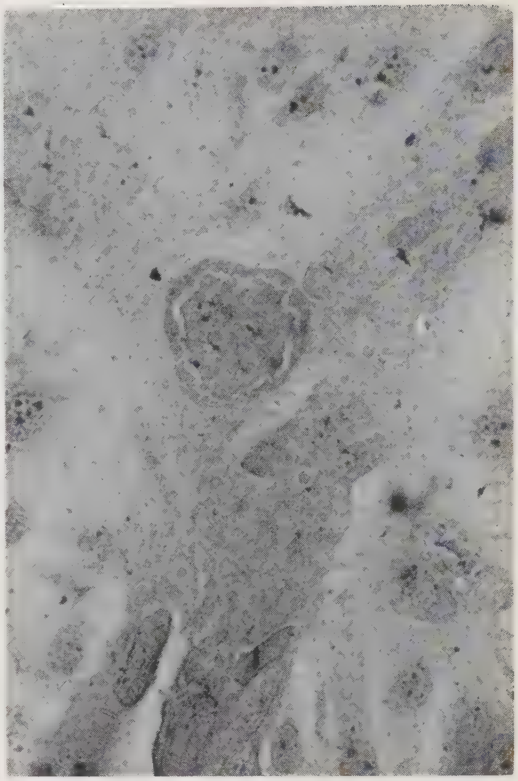
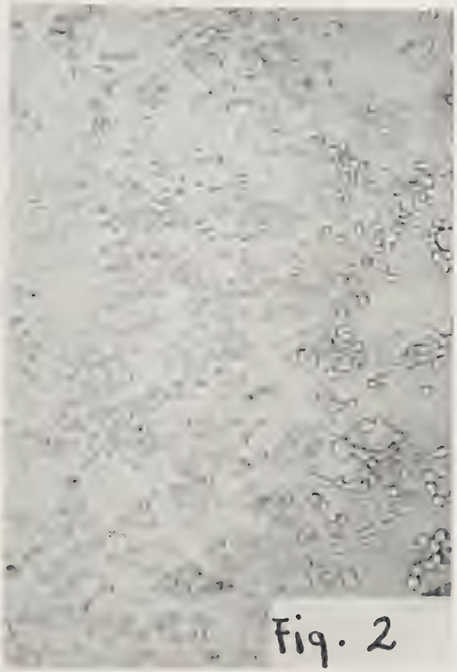


FIG. 1. Intranuclear inclusion bodies and ballooned nucleus . H & E stain × 600.

(a) *Rounding degeneration, "G" for granulation.* The initial plaque was characterized by an area of rounded and ballooned cells. Occasionally, 3-dimensional proliferation could be seen. An inspection of individual cells revealed various stages of development of type A intranuclear inclusion bodies (Fig. 1). In the earlier stages the changes in the nucleus in which there were enlargement, margination of chromatin and presence of an inclusion body of the "full" type were similar to those described by Scott *et al.*(3) in rabbit



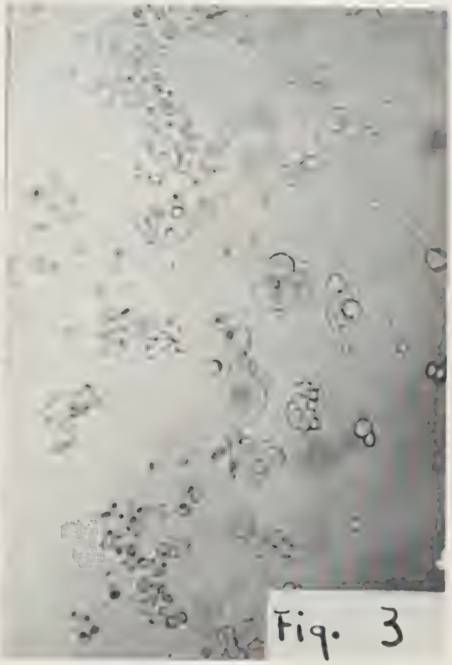


FIG. 2. Rounded cells of "G" virus, unstained × 60.

FIG. 3. Giant cells of intermediate size, unstained × 60.

FIG. 4. Aggregated ballooned nuclei with full inclusion bodies in the center of a lysed area. Cell membranes not visible, unstained × 60.

FIG. 5. Normal monkey kidney cell tube culture, unstained × 60.

TABLE I. Method of Separation of "L" Strain by Selective Limiting Dilutions.

	Passages						
Dilution	1	2	3	4	5	6	7
10^{-0}	GGGG	GGGG	GGGG	GGMM	GGGG	MLLL	LLLL
10^{-1}	GGGG	MMMM	MMML*	MMMM	MMMM	LLLL	LLLL
10^{-2}	MMMG	MMMM*	M000	MMML	MMMM	LL*00	0000
10^{-3}	MMMM	0000	0000	MMML*	MMML*	0000	0000
10^{-4}	GMMM*	0000	0000	GM00	0000	0000	0000
10^{-5}	GG00	0000	0000	0000	0000	0000	0000
Incubation, days	4	2	2	3	3	2	2
No. of nuclei in giant cells	8	10	30	50	Aggregated nuclei, cytolysis 100 or more		

* Used for passage.

G—G virus type; L—L virus type; M—mixed type.

corneal cells infected with herpes simplex virus. The inclusions seen in the later stage were typical type A, such as those observed by Traub in pseudorabies virus infection(4). Frequently the picture of an amitotic division of the nucleus was observed. With advancing infection, the rounded cells became necrotic and sloughed off from the glass. The changes finally involved the whole culture (Fig. 2).

(b) *Giant cells and cytolysis, "L" for lysis.* This reaction appeared to evolve with passage, and although giant cells were seldom absent altogether, they were more frequent in the earlier passages in which lysis was less marked than they were in the later passages in which lysis was the predominant characteristic. In the early passages there appeared among the rounded cells small giant cells each with 4-10 nuclei containing full inclusion bodies. These sometimes were connected to each other by protoplasmic strands (Fig. 3). In other places a giant cell formed the center of an isolated group of rounded cells.

As selective passages were made larger giant cells were observed containing 100 or more nuclei. Finally the characteristic picture consisted of a disappearance of cell boundaries leaving in the center a large aggregation of nuclei each containing an inclusion body, or the lytic effect of the virus was so great that only holes in the cell sheet were seen without even the nuclei remaining intact (Fig. 4). The appearance of normal monkey kidney cells is shown in Fig. 5.

Separation of 2 virus strains. This was accomplished by selective passage at limiting dilutions.

Table I summarizes the results of selective limiting dilutions. It can be seen that in the original material, which was derived from a bottle culture in which both types of cytopathogenicity were noted, the "G" strain predominated. A tube with this characteristic was then passed serially. This strain was found consistently to cause the characteristic rounding and granulation effect with no or only occasional giant cells for 25 passages. The "L" strain was obtained with more difficulty. Since no lysis was observed in the original material, those tubes containing a number of giant cells were used for passage. As mentioned, the giant cells in the earlier passages were small to moderate in size containing 8-10 nuclei. However, with passage larger and larger giant cells were seen. Lytic areas were first noticed in passage No. 3 in which the giant cells contained about 30 nuclei. Lysis appeared in all tubes of the 10^{-1} dilutions of passage 6, at which time no giant cells, as usually pictured, were seen; however, aggregations of more than 100 nuclei without definitive cell boundaries were seen in the midst of an area of lysis. By the seventh passage mainly lytic areas were noted. This characteristic was maintained for 15 further passages at limiting dilutions without change. Both "G" and "L" strains were neutralized by specific antiserum after a total of 25 passages in tissue culture.

It was also possible to separate these 2 strains by the plaque technic(2) using plasma clot overlay.

Growth curve. This was studied in both strains and little difference between the 2 was

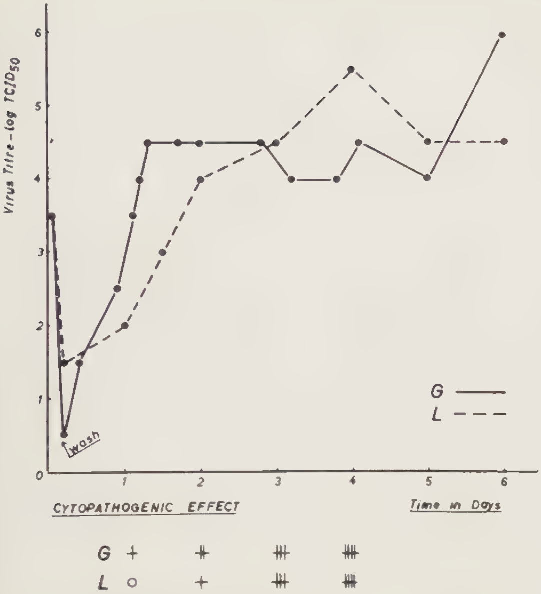


FIG. 6. Growth curve of "G" and "L" strains.

noted (Fig. 6). The highest titers were $10^{6.0}$ and $10^{5.5}$ $TCID_{50}$ respectively for the "G" and "L" strains. There was no significant difference in heat stability between the 2 strains.

Plaque Form. In order to demonstrate the difference in plaque form of the 2 strains the technic of limiting the infection to one growth cycle by means of immune serum (Black and Melnick(5)) was utilized.

One hundred plaque forming units of "G" and "L" strains of the 15th tissue culture passage were introduced into separate tube cultures. After one hour the medium was replaced with fresh growth medium containing 1:30 and 1:60 dilutions of antisera or with fresh growth medium alone. By the fourth day of incubation at 37°C the cells in the control tubes (without antiserum) of both strains had sloughed off the glass. In the tubes with 1:30 dilution of antiserum the formation of daughter foci had been completely prevented. While in the tubes with 1:60 dilution only partial prevention was obtained. The tubes with 1:30 antiserum (Fig. 7) stained with Giemsa *in situ* showed that the number of plaques was approximately equal (± 30) for each strain, indicating a similar adsorption efficiency, but that the "L" strain plaques were large, whereas the "G" strain plaques were small.

Infectivity in rats. Pseudorabies virus causes characteristic neurological symptoms in infected rats. The animals, after an incubation period of 48-72 hours after inoculation into the eye began to scratch the skin of the face on the side of the inoculated eye. The itching becomes intolerable and the animals may tear off their skin before they die. The mechanism of these symptoms has been described by Dempsher *et al.*(6). These workers found bursts of spontaneous electrical discharges occurring first in the postganglionic fibers of the superior cervical ganglion, then in both pre- and postganglionic fibers and finally only in preganglionic fibers in animals with symptoms. It was of interest to test whether there was any correlation between the type of cytopathogenicity and the production of symptoms in rats. Preliminary experi-

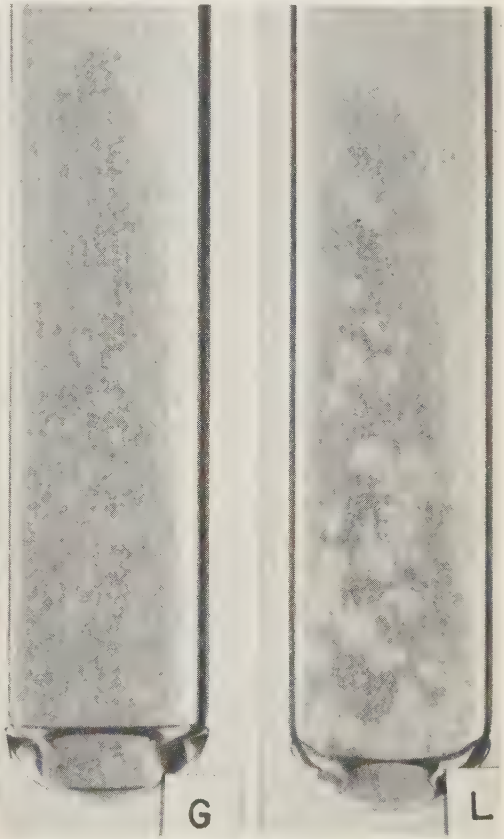


FIG. 7. Large and small plaques of "L" and "G" strains in an immune environment. Monkey kidney cell culture stained with Giemsa × 2.

TABLE II. Characterization of the Virus Strain.

	"G" strain	"L" strain
Plaque size	small	large
Cytopathogenicity	rounding cells	cytolytic giant cells
Intranuclear inclusion	positive	positive
"Mad-itch" in rat	negative or weak	”
CAM	typical pocks	typical pocks
Hemolytic activity	negative	negative
Antiserum	neutralized	neutralized

ments revealed that the "L" strain uniformly produced symptoms but these were only rarely produced by the "G" strain even though the $TCID_{50}$ titers were approximately the same.

The characteristics of the 2 strains are summarized in Table II.

Discussion. The demonstration of 2 types of cytopathogenicity in monkey kidney cells infected with pseudorabies virus suggests either that the original stock virus contained 2 strains of virus particles which could be differentially selected by adaptation in monkey kidney cells or that mutation from the "G" to the "L" strain occurred in this host. At present it has not been possible to separate these 2 strains sufficiently cleanly to test them for genotypic characteristics by recombination experiments. The apparent correlation between type of cytopathogenicity and ability to cause symptoms in rats raises the possibility that the virus which affects cell membranes in tissue culture leading to lysis may have some action on the nerve membrane which in turn gives rise to the abnormal electrical responses described by Dempsher *et al.* This point is under further investigation.

Summary. During the course of adaptation of the egg-adapted pseudo-rabies virus to tissue culture of monkey kidney cells, 2 types of cytopathogenic variations were encountered which appeared to be induced by 2 different strains of virus. One strain produced rounding degeneration, and the other cytolytic giant cell formation. Descriptions are given of the growth curve, plaque type in tissue culture, and infectivity for rats of these 2 strains.

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Influence of Estrogens on Body Growth and Food Intake.[*] (23393)

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Estrogens have been shown by many workers to depress body weight in laboratory animals(1-5). Several lines of evidence, too extensive to review here, indicate that they may do this by influencing either the release or action of other hormones in the body. Heavy or prolonged treatment with estrogens also causes anorexia. The resulting inanition may be partially responsible for the effects sometimes attributed to hormonal alterations. In addition, inanition itself may result in hormonal imbalances(6,7,8), which resemble in some respects the effects brought about by estrogen treatment. Therefore, dietary restriction, whether imposed experimentally or occurring as a result of estrogen treatment, may act on growth directly as well as indirectly through alteration in endocrine functions.

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Growth depression in rats, accompanied by voluntary restriction of food intake as a result of estrogen treatment has been most consistently demonstrated with stilbestrol. The depression of growth and food intake was proportional to the dosage of stilbestrol; growth depression could be duplicated by pair-feeding(1). After a single dose or repeated administration of estrogen, there is a negative nitrogen balance attributable to decreased intake(2,3). Estradiol, or the benzoate, 100 μg daily, has been reported to produce either no body weight depression(4) or depression without influence on food intake(1). It thus appeared that in rats the natural estrogens, represented by estradiol, were less effective than stilbestrol in depressing body growth, although the 2 compounds have approximately the same activity in the Allen-Doisy test(9).

Since the effects of estradiol on body growth and food intake reported in the literature have been quite variable, a range of doses was first explored to determine if a dose of this natural estrogen could be found that would produce statistically significant depression of growth and food intake. In later and more extensive experiments, growth rates of estradiol-treated rats were compared with those of pair-fed controls. Results indicate that estradiol produces a significant depression of growth, which can be readily duplicated by restricting food intake of the controls to that of the estrogen-treated partner.

Materials and methods. Male rats of the Sprague-Dawley strain,[‡] weighing less than 80 g at the start of the experiments, were divided into groups of 3 to 4 animals and injected subcutaneously with estradiol, in 0.1 ml sesame oil, in doses of 1, 10, or 100 μg per rat daily, or with estradiol benzoate in doses of 2, 20, or 200 μg per rat every other day, control animals receiving equal volumes of the vehicle.[§] The animals were housed in individual wire-bottomed cages at 70°F, and fed a diet of ground Ralston-Purina chow checkers. Food was presented in tared metal cups secured to the cages to eliminate tipping, and fitted with an anti-scatter cover.[‖] When pair-feeding regimens were undertaken, each treated rat was matched with an untreated rat of similar body weight. The pair-fed control was presented throughout with the amount of food eaten *ad lib.* by the treated member during each previous 24-hour period. Records of body weight and food consumption were made daily each morning. Mean body weight and food consumption were calculated for each group, and the significance of the difference of the means tested by application of "Student's" t test.

Results. In preliminary experiments with estradiol, a significant depression of body growth, proportional to dose, was found for all groups after 30 days' treatment. The differences among the doses of hormone did not, however, prove significant. At 30 days a significant depression of food intake was also observed in the groups on the 2 highest doses, while a suggestive depression occurred with the lowest dose ($P>0.05<0.1$). Body weight depression was proportionately greater than food intake, the amount of the disproportion being directly related to the dose. When the control animals were pair-fed with rats treated with the highest dose of estradiol (100 μg daily), their growth rate tapered off to parallel that of the treated rats. Restoration of *ad libitum* feeding led to an immediate resumption of growth. Estradiol benzoate produced similar effects on growth, food intake, and efficiency, but they were prompter and more clear-cut than with estradiol. For this reason; all subsequent experiments were conducted with estradiol benzoate.

Growth rates of the estrogen-treated rats did not differ from those of the untreated, pair-fed controls, whether pair-feeding was begun at the start or in the middle of the experiment

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[‡] Purchased from the Charles River Breeding Laboratories.

[§] Estradiol was obtained through the generosity of the Schering Corp., Bloomfield, N. J., the estradiol benzoate as a gift from Ciba Pharmaceutical Products, Summit, N. J.

[‖] Purchased from Geo. H. Wahmann Manufacturing Co., Baltimore, Md.

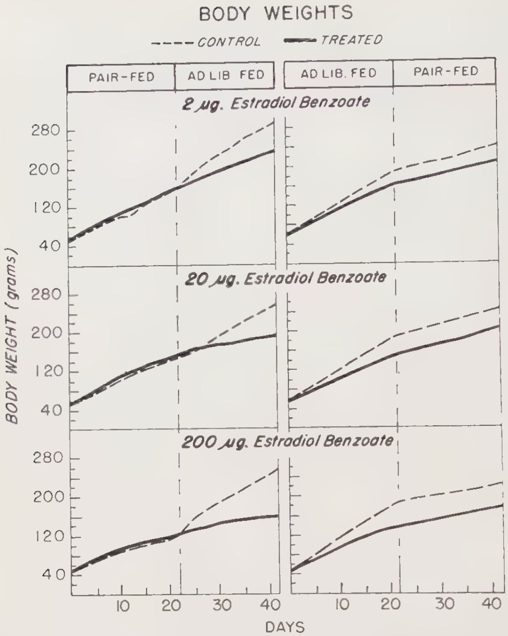

FIG. 1. Action of estrogen on body wt under conditions of *ad lib.* restricted pair-feeding.

(Fig. 1). Experiments in which the controls were initially fed *ad libitum* (right aspect, Fig. 1) demonstrated depression in body weight gain of the estrogen-treated rats proportional to the dosage of estrogen, which was significantly different from the controls with the 2 higher doses at the end of 3 weeks treatment. Placing the controls on restricted pair-feeding resulted in their growth rates paralleling those of the treated rats, at each level of estrogen dosage. Similarly, growth of the treated rats and their respective controls for each estrogen dosage were identical when pair-feeding was begun initially (left aspect, Fig. 1). When the controls were allowed to resume *ad libitum* feeding, their growth rates immediately accelerated, while those of the estrogen-treated rats remained unchanged. By the end of 3 weeks, the body weights of the estrogen-treated rats were now significantly less than those of the controls, and the extent of these differences was proportional to the dosage of estrogen. Voluntary food intake of the estrogen-treated animals was consistently less than that of the untreated controls eating *ad libitum* (Fig. 2). At 3 weeks, the differences were significant for the highest dose of estrogen for both initial and delayed *ad lib.* feeding, and for the intermediate dose for initial *ad lib.* feeding. Depression in body weight was proportionately more marked than that of food intake for the estrogen-treated groups compared to the controls fed *ad libitum,* as found previously with estradiol.

In one group of experiments, the relative influence of estrogen and of the pair-feeding restriction on gonads and sex accessories was also determined by weighing ventral prostate, seminal vesicles, and testes at the end of an experiment (Table I). As would be expected after chronic treatment, estrogen led to depression roughly proportional to dose for prostate and testes. While pair-feeding brought about depression of the weight of sex-accessories, it was proportionally not as great as that obtained with estrogen. Testicular weight, however, was not affected by restricted feeding.

Discussion. Estradiol and its benzoate in adequate doses brings about a profound depression in growth rate accompanied by voluntary restriction in food intake. The failure of similar doses of estradiol benzoate (100 µg

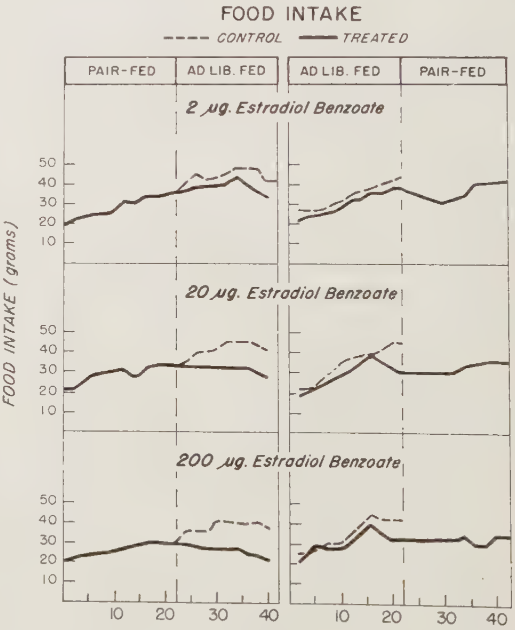

FIG. 2. Influence of estrogen dosage on food intake.

TABLE I. Effect of Estrogen or Pair-Feeding on Testes and Sex Accessories.

	Organ weights					
	Prostate		Seminal vesicles		Testes	
Group	mg absolute	Per 100 g body wt	mg absolute	Per 100 g body wt	mg absolute	Per 100 g body wt
Ad lib. controls	309	100.8	714	230	3100	978
2 μg estrogen	53	24.4	93	44.2	826	372
Pair-fed	195	71.8	553	206	3363	1345
20 μg estrogen	17	8.8	124	64.4	258	134
Pair-fed	166	66.6	311	123.3	3030	1200
200 μg estrogen	11	6.1	137	80.6	210	124
Pair-fed	202	88.2	539	232	2950	1285

every other day for 3 weeks) to influence body weight in 200 g male rats in the experiments of Lynch(4) may be due in part to the use of older, heavier animals, which are probably less sensitive to the growth-depressant action of this estrogen than younger, more rapidly-growing rats. Furthermore, when estrogen dosages are calculated on the basis of body weight, his dose is about equivalent to the intermediate dose used in the present experiments.

Differences in age, weight, and possibly sex may also explain the divergence of results obtained in the present experiments from those reported by Meites(1), who did not find any reduction in growth rate until after 30 days treatment and no decrease in food intake regardless of the length of treatment in 150 g female rats given 100 μg of estrone or estradiol daily. The nature of the diet, perhaps quite crucial, was not stated. Under the conditions of our experiments we could not confirm this author's conclusion that "growth reduction can be induced with natural estrogens without lowering daily food intake."

Both the present experiments with estradiol or estradiol benzoate, and the experiments of Meites(1) with diethylstilbestrol have shown that pair-feeding duplicates the effects of estrogens on body weight. Somewhat different results were obtained with larger doses of diethylstilbestrol by Glasser(2) who found that in 250-350 g adult male rats, fed a semisynthetic diet containing 18% casein, daily injection for 3 weeks depressed average body weight 100 g, while pair-feeding depressed body weight only 55 g. Rats receiving stilbestrol showed at the same time a greater negative nitrogen balance than pair-fed controls. With further treatment, the depression of growth and food intake tapered off and return toward normal of the negative nitrogen balance occurred. A similar phenomenon was reported by Selye(5) who noted an initial weight loss during the first week followed by a slow gain in rats given excessive daily doses of estradiol or stilbestrol. This secondary recouping of weight loss, termed "adaptation" by Selye was not seen in the present experiments; this does not exclude the possibility that adaptation might have been detectable in nitrogen balance studies. No stimulation of body weight gain or increase in food utilization efficiency, similar to that reported for cattle(10,11) or sheep(12) treated with stilbestrol, has in our knowledge been reported to occur in small laboratory animals. The interpretation that voluntary restriction of food intake was chiefly responsible for the negative nitrogen balance in the experiments cited above(2,3) is strengthened by the present results with estradiol and those of Meites(1) with stilbestrol.

Although pair-feeding failed to reproduce the inhibitory action of estrogen on the hypophyseal-gonadal system, the fact that estrogen produces general effects on growth and appetite suggests that the effects of chronic estrogen treatment on other endocrine systems and skeletal growth should be reinvestigated in experiments using pair-fed controls.

Summary. The effects of estradiol and its benzoate ester on body weight and food consumption of immature male rats have been studied in experiments using controls which were either eating *ad lib.* or pair-fed to the

treated animals. In animals eating *ad lib.*, estrogen treatment for 3 weeks or more resulted in a depression of body weight accompanied by a voluntary restriction in food intake; body weight was more greatly depressed than food intake. Restriction of food intake in the controls to amounts eaten *ad lib.* by the treated animals duplicated the effects of the estrogen on depression of body weight, since the growth curves of pair-fed control and treated groups became parallel.

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Free Amino Acid Patterns of Certain Tissues from Potassium-Deficient Dogs.* (23394)

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Rats placed on potassium-deficient diets, with or without simultaneous administration of desoxycorticosterone acetate (DCA), exhibit typical changes of the electrolyte compositions of plasma and skeletal muscle(1,2). Thus, there is an increased plasma bicarbonate content and a lowered plasma chloride concentration in association with a loss of skeletal muscle potassium and an unequivalent gain of muscle sodium. Cooke *et al.*(3) developed the thesis that the unequivalent distribution of potassium and sodium is accompanied by a passage of hydrogen ions into the muscle cell, thus accounting for the alkalosis of the extracellular fluid. Dogs fed a potassium-deficient diet, with or without simultaneous administration of DCA, also exhibit a loss of skeletal muscle potassium and an unequivalent gain of sodium. However, the increased plasma bicarbonate content and fall of the plasma chloride concentration become conspicuous only with simultaneous dietary restriction of chloride(2,4).

Evidence was recently presented(5), on the basis of paper chromatography, that the amino acids lysine and arginine were increased in the skeletal muscle, diaphragm and kidneys from potassium-deficient rats, while simultaneously there appeared to be decreased concentration of the amino acids aspartic acid and glutamic acid in these same tissues. The suggestion was made that possibly the changes in the concentrations of basic and acidic amino acids in the skeletal muscle of the rat may compensate in part the discrepancy between the potassium loss and sodium gain. This view was supported by the observations of Eckel, Pope and Norris(6) that from 8-40% of the metallic cation deficit present in the potassium-depleted rat skeletal muscle could be accounted for by the amino acid lysine.

The results with rats prompted the present study of the free amino acid patterns of skeletal muscle, left ventricle and kidneys from potassium-deficient dogs. Special attention was given to potassium and sodium concen-

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trations and free amino acid patterns of skeletal and heart muscle in relation to changes of plasma chloride and bicarbonate concentrations.

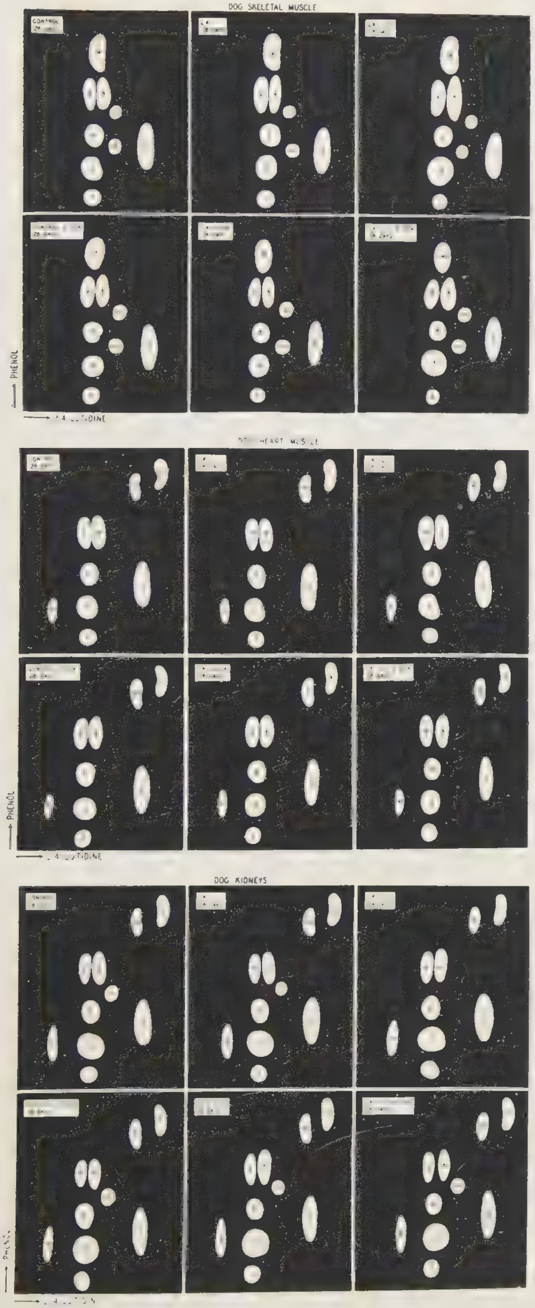

FIG. 1, 2 and 3. Free amino acid patterns of skeletal muscle, left ventricle and kidney. Numbers within the tracings indicate the following substances: 1, aspartic acid; 3, glutamic acid; 4, lysine; 5, taurine; 6, glycine; 8, glutamine; 9, alpha-alanine; 10, valine; 11, leucine; 12, serine; 13, threonine; 14, carnosine.

Methods. Young adult mongrel female dogs were treated in exactly the same manner as described previously(4). The animals given DCA received 0.5 mg DCA (Percorten, Ciba Pharmaceutical Products, Inc.) per kilo body weight per day by subcutaneous injection. At the close of the given dietary period, the animals were placed under sodium pentobarbital anesthesia for collection of blood and tissues. The sample of skeletal muscle (lumbar portion, sacrospinalis), the kidneys and the heart were quickly excised, blotted free of adhering blood, and cleaned of capsulae or connective tissue. An aliquot of the tissues was quickly weighed and immediately placed in cold (0°C) tungstic acid (1 part tissue plus 9 parts tungstic acid (prepared in the ratio of 1 part 10% sodium tungstate: 1 part ⅔ NH_2SO_4:7parts H_2O)), homogenized, and filtered in the cold (0°C). One-tenth ml aliquots of these tungstic acid filtrates were used directly for paper chromatography, utilizing the same technic as in previous experiments with rat tissues(5), employing as developing solvents phenol (80%) and 2,4 lutidine (65%). The methods for chemical analyses of plasma, skeletal muscle and left ventricle were the same as previously described(4).

Results. Fig. 1, 2, and 3 show the average tracings of the chromatograms obtained with tungstic acid filtrates of skeletal muscle, left ventricle and kidney from normal and potassium-deficient dogs. In Table I are presented the electrolyte data for plasma, skeletal muscle and left ventricle.

For a given tissue (skeletal muscle, left ventricle and kidney) the substances identified qualitatively with the volume of tungstic acid filtrate used in spotting the chromatograms were the same in the control and potassium-deficient dogs (Fig. 1,2,3). From previous experience with rat tissues(5), the absence of a difference between the chromatograms in the control and experimental dogs, particularly with respect to skeletal muscle, came as a surprise. In the case of rats, the conspicuous changes in skeletal muscle with

TABLE I. Average Electrolyte Composition of Plasma, Skeletal Muscle, and Left Ventricle.

Diet*	Plasma†		Skeletal muscle		Left ventricle	
	HCO_3	Cl	Na	K	Na	K
			mEq			
Control	24.8	116.8	8.6‡	37.8‡	15.6	41.1
" + DCA	25.7	113.2			15.2	39.0
Low K	20.3	122.8	10.3‡	33.8‡	15.1	40.6
" + DCA	31.7	108.5	14.3‡	26.5‡	14.7	37.6
" & low Cl	36.5	101.8	13.9‡	34.2‡	18.9	37.5
Idem + DCA	43.8	96.6	30.5	30.8		

* Dietary period of 28 days. Two dogs in each group.

† Plasma values are given in terms of 1000 g water, and muscle values in terms of 100 g fat-free solids.

‡ Skeletal muscle data from comparably treated dogs reported previously(4).

a cellular potassium loss was a gain of the amino acids lysine and arginine and a tendency toward a loss of the amino acids glutamic and aspartic. In the dog similar changes were anticipated, particularly in the animals fed the low-potassium or low-potassium and low-chloride diets, with or without simultaneous administration of DCA, since the skeletal muscle exhibited a lowered potassium content under these circumstances (Table I).

Discussion. No explanation can be given for the difference between the free amino acid patterns of skeletal muscle from potassium-deficient dogs and those previously reported (5) for skeletal muscle from potassium-deficient rats. The present findings do not exclude the possibility that the conditions under which the extraction of amino acids was effected in rat tissues are unsuitable for dog tissues. However, the fact that the pattern of identifiable amino acids in the skeletal muscle from the 2 species is identical argues against the observed difference being attributable to the method of amino acid extraction. The difference also appears unexplainable on the basis of smaller electrolyte changes in dog plasma or skeletal muscle. For example, the dogs fed the low-potassium and low chloride diet and given DCA exhibited marked alterations in the electrolyte compositions of both the plasma and skeletal muscle (Table I), yet the free amino acid pattern was identical with that of the control.

The significance of the alterations of apparent concentrations of basic and acidic amino acids with respect to the mineral cation deficit observed in skeletal muscle from potassium-deficient rats requires further study. Additional experiments involving quantitative measurement of free amino acids are required to evaluate this point, and, such data may help to explain the differences observed between rats and dogs.

Summary. Tracings of the amino acid patterns obtained chromatographically on the tungstic acid acid filtrates of skeletal muscle, left ventricle and kidney from normal and potassium-deficient dogs have been presented. The amino acid patterns of the tissues from control and potassium-deficient dogs were found to be identical, even in the presence of marked changes in the electrolyte compositions of plasma and skeletal muscle. Differences between amino acid patterns of the skeletal muscle from potassium-deficient dogs and of the skeletal muscle from potassium-deficient rats previously reported were pointed out.

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Effects of Animal Sera and Serum Albumin on Latex-Fixation Test for Rheumatoid Arthritis.* (23395)

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The recently introduced latex-fixation test (1,2,3) provides a relatively simple and easily performed technic for the serologic study of rheumatoid arthritis. Thus it has some advantages over the more cumbersome, but widely employed, sheep cell agglutination tests(4,5,6,7).

Despite the technical problems associated with these latter procedures, they have proven invaluable for investigation of the factor or factors first reported by Waaler(8) in rheumatoid sera responsible for enhancing agglutination of sensitized sheep erythrocytes.

The use of human gamma globulin in the Heller FII test(5) and rabbit anti-sheep erythrocyte serum as the "antigen" source in the sensitized sheep cell test is well established. Others have reported guinea pig and horse sera also to be adequate(9,10).

It is the purpose of this report to show that, similarly, sera from several animal species can be substituted for human gamma globulin in the latex-fixation test. Furthermore, experiments will be described in which "antigen" reactivity of test sera was gained by either removal of albumin by Na_2SO_4 precipitation or by dilution.

Materials and methods. Latex-fixation test. The basic test as employed by these laboratories has been described previously (3) and is essentially the same as that suggested by Singer and Plotz(1). Human gamma globulin is mixed with a borate-buffered saline (pH 8.2) to which is added a measured quantity of latex particles. Two drops of this mixture are brought into the presence of 2 drops of the test serum dilutions (1:20 through 1:5120). After incubation for 30 minutes at 37°C or 56°C (water-bath), the tubes are centrifuged lightly and examined for evidence of flocculation. *Sera.* The human sera employed in this study were obtained for the Rheumatology Clinic of the University Hospital. Animal sera were collected at the Department of Bacteriology and from the clinics of the College of Veterinary Medicine. *Globulin.* In addition to the human gamma globulin (poliomyelitis immune globulin, 150 mg per ml) routinely used in these laboratories, porcine globulin commercially prepared by Armour and Co. was studied. *Serum fractionation.* Sera were fractionated according to the technic of Thurston, *et al.*(11). This is a Na_2SO_4 precipitation which required but small quantities of sera (1.0 ml or less). Albumin is effectively removed with this technic and no dialysis is necessary.

Results. Animal globulins as source of "antigen" in latex-fixation test. Nine sera from a variety of animal species were tested as possible sources of "antigen" by the latex-fixation technic. One-half ml of undiluted human or animal serum was substituted for 0.5 ml of 0.5% human gamma globulin in the preparation of the latex "antigens" (Table I). Each of these "antigens" then was tested with 1:20 dilutions of 3 standard sera: A, a strongly reactive rheumatoid serum; B, a rheumatoid serum of moderate reactivity; and C, a normal human serum. No flocculation was observed except in 2 instances (chicken serum and dog serum) and these proved to be non-specific inasmuch as the saline controls also showed evidence of flocculation. The sera from several chickens reacted similarly, while no other dog serum was tested.

Subsequently 1.0 ml of each serum was fractionated with Na_2SO_4 and 0.5 ml of the reconstituted globulin was used to prepare latex "antigens" as before. After ascertaining that the "antigens" were reactive with 1:20 dilutions of the standard sera, titrations were performed and the results compared

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TABLE I. Serum Globulins from Various Species as Source of "Antigen" in the Latex-Fixation Test.

Latex antigen prepared with:				Titer of standard sera, initial dilution 1:20		
				Rheumatoid arthritis		Normal
Whole serum	Reconstituted globulins	Gamma globulin	Saline control	A	B	C
Human			—	—	—	—
Rabbit			—	—	—	—
Porcine			—	—	—	—
Chicken			4+	4+	4+	4+
Guinea pig			—	—	—	—
Dog			1+	±	±	±
Cat			—	—	—	—
Horse			—	—	—	—
Bovine			—	—	—	—
Sheep			—	—	—	—
	Human		—	*5120	160	—
	Rabbit		—	1280	40	—
	Porcine		—	5120	80	—
	Chicken		4+	- - - - -	- - - - -	4+
	Guinea pig		—	640	40	—
	Dog		±	- - - - -	- - - - -	1+
	Cat		—	1280	160	—
	Horse		—	1280	80	—
	Bovine		—	640	80	—
	Sheep		—	1280	80	—
		Human	—	5120	160	—
		Porcine	—	2560	80	—

* Titers expressed as reciprocals of serum dilutions.

with those found by the conventional technic (see human gamma globulin). Although some variation in titers was noted, globulins from all species, other than chicken and dog, as noted previously, functioned as "antigen." Whether or not the variation in reactivity (3 tube dilution range) reflects quantitative differences in the "antigen" content of the sera was not determined. More likely, the precipitation procedure provided the source of variation since this technic was reported with rabbit and human sera only. Adjusting the percentage of the precipitant (Na_2SO_4) may have corrected these discrepancies.

The purified porcine and human gamma globulins behaved similarly as "antigens."

Diluting human sera as a means of gaining "antigen" activity. The results just described suggested that the albumin in undiluted sera, in some manner, interferes with the mechanics of the latex-fixation test since removal of the albumin by precipitation permitted the "antigenic" properties of the globulins to be demonstrated.

Ten human sera from apparently normal individuals were diluted by 2-fold increments from 1:20 through 1:320 with borate-buffered saline (pH 8.2). One-half ml of each dilution was used in the preparation of latex "antigen" as before. "Antigen" prepared from the undiluted serum was included in each "antigen" series. Two drops of a strongly reactive rheumatoid serum were mixed with an equal quantity of each "antigen" in all 10 series. After incubation, the resulting flocculation was recorded as 4+, 3+, 2+, 1+, ± or — (Table II). Since 9 of the 10 sera demonstrated reactivity as "antigens" when diluted, it seems probable that the interfering capacity of albumin is, at least in part, a function of its concentration. Diluting the majority of sera depreciated this property without completely eliminating the activity of the globulins. Differences in activity were noted among the series, as might be anticipated.

A similar experiment conducted with the animal sera listed in Table I yielded analogous results.

Singer and Plotz(1) reported that the con-

TABLE II. Dilution of Serum as a Means of Gaining ''Antigen'' Activity.

Normal human serum No.	Reactivity of positive rheumatoid serum tested with latex antigen containing dilutions of normal human sera				
	Undil.	1:20	1:40	1:80	1:160
1	—	—	—	—	—
2	—	1+	1+	±	—
3	—	2+	1+	—	—
4	—	±	1+	±	—
5	—	1+	±	±	—
6	—	±	1+	±	—
7	—	1+	1+	±	—
8	—	±	1+	±	—
9	—	1+	2+	±	—
10	—	±	—	—	—

centration of gamma globulin employed in the latex-fixation test could be varied over a wide range. Concentrations above 12,500 γ interfered with the test, while quantities of less than 25 γ failed to be fully reactive. The working concentration recommended by these authors is 250 γ of globulin per ml of latex-globulin antigen.

The following experiment was conducted (1) to determine if the same minimal concentration of globulin (25 γ) would be effective with the "drop" modification of the latex-fixation test employed here, and (2) to correlate this finding with the results of titrating the reactivity of human sera (Table II).

Three strongly reactive rheumatoid sera (I, II, III) were tested by the "drop" technic with latex antigens containing 4-fold decreases in globulin concentration as indicated in Table III. The results were in agreement with those described by Singer and Plotz(1), *i.e.*, maximum reactivity was observed when the globulin concentration of the "antigen" approximated 250 γ per ml. However, some reactivity resulted with "antigens" containing as little as 3.9 γ of globulin.

Since 8 of the 10 human sera demonstrated some level of "antigenicity" when diluted at 1:40 (Table II), the concentration of globulin in this dilution was calculated from data available in the Handbook of Biological Data (12). The figure of 11.2 γ is in keeping with the results shown in Table III. Seven of the 10 human sera reacted, although minimally, in even greater dilutions (1:80), which gamma globulin concentration also would correspond approximately to the least reactive quantity noted with the more pure globulin fraction (3.9 γ).

Depressant effect of gelatin on reactivity of human gamma globulin as "antigen" in latex-fixation test. The inhibiting effect of albumin was assumed to be due to its non-specific protective colloid properties. Since this property is also associated with gelatin and may be imparted or demonstrated simply, this substance was employed in the following tests.

Bacto-Gelatin was diluted in borate-buffered saline (pH 8.2) to 5.0% concentration by warming at 56°C. The working concentration of human globulin (0.5%) was prepared by mixing 0.5 ml of the original globulin stock (150.0 mg per ml) with 14.5 ml of the gelatin solution. The latex-globulin "antigen" containing 0.5 ml of this globulin-gelatin mixture was added to buffered saline and latex in the usual manner.

Eleven rheumatoid sera of differing reactivities and 4 negative sera (as measured with the conventional latex "antigen") were diluted 1:20 and screened with the new antigen. No reactivity was observed, thus substantiating the premise that a protective colloid can interfere with the "antigenicity" of globulin. Identical results were obtained with 3.0% gelatin.

One drop of each of the diluted sera was mixed with one drop of 5.0% gelatin. The resulting 1:40 dilutions of sera were tested with the conventional latex-globulin "antigen." For purposes of comparison, 1:40 dilutions of the same sera were prepared in saline only and examined simultaneously. The results (Table IV) illustrate the depressant effect of gelatin on the rheumatoid factor.

The influence of diluting the sera initially

TABLE III. Titers of Rheumatoid Sera Tested with ''Antigens'' Containing Decreasing Concentration of Globulin.

Serum No.	Concentration of globulin (γ) per ml of latex antigen				
	250	62.5	15.6	3.9	1
I	5120*	2560	2560	640	—
II	5120	5120	5120	1280	—
III	5120	5120	1280	80	—

* Titers expressed as reciprocals of serum dilutions.

TABLE IV. Depressant Effect of Gelatin on Reactivity of Rheumatoid Sera in Latex-Fixation Test.

Test serum No.	Reactivity when diluted 1:40 in: Saline	Equal parts of saline and 5% gelatin
1	3+	±
2	4+	1+
3	2+	—
4	3+	2+
5	3+	1+
6	1+	—
7	1+	—
8	2+	—
9	4+	±
10	2+	—
11	2+	—
12	—	—
13	—	—
14	—	—
15	—	—
16	—	—

with gelatin on titration endpoints also was tested. The gelatin influence was dissipated by the serial dilutions in saline since the titers were not materially affected, depending, of course, on the original reactivity of the serum being investigated.

Viscosity measurements of "antigens" prepared with and without gelatin failed to reveal differences which could account for the observed property of gelatin described above.

Discussion. The sensitization of erythrocytes with antibody-containing sera from several animal species, prior to demonstrating the agglutinability of such treated red cells upon admixture with rheumatoid sera is of considerable interest. Although not elucidating the component of serum globulin responsible for the "antigenicity" conferred on the sensitized red cells, the ubiquity of this component is established. In all such procedures, the albumin content of the anti-erythrocyte serum is of no consequence since this fraction is devoid of antibody and would not enter into the sensitization mechanism. The coating of tanned erythrocytes with the FII fraction (gamma globulin) of human serum also eliminates any possible influence of albumin in the Heller test(5). Similarly, employing human gamma globulin as the source of "antigen" in the more conventional latex-fixation technics (1,2,3) also excludes this factor from consideration.

In the studies discussed here, the sera of 7 animal species, in addition to human, were shown to contain the necessary "antigenic" component. The demonstration of this component, however, was dependent on the removal of albumin, either by precipitation procedures or by dilution. The fact that various animal sera can provide the "antigenic" component for the latex-fixation test likens it to the findings noted with the sensitized sheep cell technics.

The failure of the whole sera to function as "antigen" was attributed to some serum component or components acting as a protective colloid and, thus, effectively prohibiting the serologic reaction from attaining completion. The recognized property of gelatin to act nonspecifically as a protective colloid was employed to establish this fact. Attempts to simulate this property by adding albumin to either diluted but reactive sera, or to reactive globulins were not uniformly successful. Further efforts in this regard were abandoned inasmuch as the pepitization procedures indicated did not seem warranted and could only serve as substantiative evidence. Since gelatin had already been shown to prohibit gamma globulin from functioning as "antigen," it would seem that the interference shown with whole serum was, at least in part, associated with a more intimate property of albumin than can be demonstrated by mere admixture.

These findings may bear some significance to the fact that the activity of rheumatoid sera is best demonstrated in the latex-fixation test, if the sera are first diluted 1:20. Since both the "antigenic" component of globulin and the rheumatoid factor are thought to reside in the same or closely related serum fractions, dilution would in effect decrease the influence of albumin and the inhibitor to the rheumatoid factor (often considered to be synonymous with the "antigen"), as well as the rheumatoid factor. Should the latter be in sufficient concentration, a serum so diluted would still be reactive when brought into the presence of globulin and latex. This is in a sense the basis for the agglutination-inhibition test of Ziff(7). Some confirmation for this lies in the report of Singer and Plotz(1) who found that 11% of the strongly positive sera reacted with latex and buffer, without benefit of added globulin.

The reactivity of adult chicken serum with latex and buffer is without explanation at present. Perhaps the relative concentration of albumin to globulin in chicken serum or the pH or ionic influence of this particular buffer is of significance. Embryonic sera did not behave in this manner.

Summary. The sera from 7 animal species were found to contain globulin components which would function as "antigen" for rheumatoid sera in the latex-fixation test. The effectiveness of these sera as "antigens" depended on removal of albumin by either precipitation or dilution. Nine of 10 human sera behaved similarly. The addition of gelatin to gamma globulin resulted in loss of "antigenicity."

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Elution of Pollen Antigens from Tannic Acid Treated Erythrocytes.* (23396)

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The use of Boyden's technic of adsorbing protein antigens(1) onto tanned red cells allowed several investigators(2-6) to measure antibody levels against pollen antigens in hayfever serum. Orlans *et al.*(2) were able to detect antibodies in the sera of treated cases and of some untreated cases, but not in the sera of non-hayfever persons. Feinberg *et al.* (6) described similar findings, but also showed that the hemagglutinating antibody detected in these hayfever sera is not identical with the reagin type of antibody. All of the previously reported work used a multi-component antigenic extract of various pollens. The present study is concerned with a crude fractionation of the whole saline extract of short ragweed pollen to reduce the number of antigenic specificities, and with the fact that some antigenic components of short ragweed extracts slowly and spontaneously elute from the surface of tanned sheep erythrocytes.

Materials and methods. Extracts of short ragweed pollen were Seitz filtered and stored as stock antigen at 10°C(6). The whole saline extract containing 116 μg protein N/ml was designated Antigen #1.

An aliquot of the whole short ragweed extract (10 ml) was treated with 4 ml of sterile, cold, 10% trichloroacetic acid (TCA) and allowed to stand at 10°C for one-half hour. The precipitate was removed, washed 3 times with cold 10% TCA, dissolved with pH 7.2 buffered saline, and diluted to the original antigen volume. This was designated Antigen #2.

The TCA non-precipitable supernate was

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TABLE I. Hemagglutination Titration Immediately Following Preparation of RBC-Antigen Complex.

		Hemagglutination of tanned-S RBC-SR								
Antigen No.	No. hrs* incubation	20	40	80	160	320	640	1280	2560	C
1	2	4	4	4	4	4	4	3	2	—
	36	4	4	4	4	4	4	3	2	—
2	2	4	4	4	4	4	3	2	2	—
	36	4	4	3	1	—	—	—	—	—
3	2	4	4	4	4	4	4	4	3	—
	36	4	4	4	4	4	4	4	2	—
4	2	4	4	4	4	4	4	2	1	—
	36	4	4	2	—	—	—	—	—	—
5	2	—	—	—	—	—	—	—	—	—
	36	—	—	—	—	—	—	—	—	—
Control S RBC without antigen	2	—	—	—	—	—	—	—	—	—
	36	—	—	—	—	—	—	—	—	—

* After the 2 hr reading, the tubes were thoroughly mixed and re-incubated for 36 hr.

recovered and placed in a dialyzing sac, aseptically. This fraction was dialyzed against 4500 ml of 0.85% NaCl solution over a 3 hour period (1500 ml/hr with constant stirring). The volume was then concentrated from 13 to 8 ml. The fluid was removed aseptically and was still TCA negative. This is Antigen #3.

Antigen #4 was prepared by digesting 2 ml of short ragweed Antigen #1 with 2 ml of twice crystallized trypsin (0.066 mg) at 37°C for 44 hrs. This solution, after the 44 hrs incubation, was inactive for digesting 1% gelatin. The digested short ragweed material was precipitated with 10% TCA, as above. The precipitate, dissolved and resuspended as above, was designated Antigen #4. The supernate, after dialysis, was concentrated and designated Antigen #5.

Rabbit anti-short ragweed serum, which contained precipitins to Antigen #1, was used in hemagglutination tests. The details of the technic are described elsewhere(6). Washed sheep RBC were treated with 1:20,000 tannic acid for 10 minutes at a pH of 7.2. The tanned cells were washed and exposed to the various antigen preparations at pH 6.4. The tanned cells with antigen adsorbed were stabilized with washes of normal rabbit serum (1:200). Titrations were performed with 2-fold serial dilutions of immune sera. Pattern readings were made after 2 hours at room temperature. The tubes were then thoroughly agitated and the tests reincubated for 36 hours, at which time the titers were again recorded.

Aliquots of the tanned sheep RBC, with the various antigen preparations adsorbed on the surface, were incubated in the cold for 36 hours. The cells were centrifuged and the supernatants were removed for skin testing in a short ragweed sensitive individual, for the presence of free antigen.

Results. Each of the 5 antigens was exposed to tannic acid treated sheep erythrocytes (S RBC) prepared with sterile precautions. To test the possibility that a polysaccharide antigen may be active, the antigens were also exposed to untanned sheep RBC (7). These various adsorbed antigen preparations were added to rabbit-anti-SR serum diluted serially. The results of the hemagglutination with the 5 antigens adsorbed onto untanned sheep RBC were uniformly negative and are not recorded. However, washes from the supernates of these tests were active in skin tests in SR sensitive individuals. Table I records the results obtained in the tannic acid type hemagglutination tests with antigens prepared as above, and stabilized with normal rabbit serum 1:200. There are 2 readings for each antigen in Table I. After reading the pattern at the end of 2 hours' incubation, the tubes were shaken and allowed to resettle for 36 hours. If antigen was eluting from the cells in sufficient quantity it should inhibit the agglutination. The data show a 16-fold decrease in titer with Antigen #2, and a 32-fold decrease with Antigen #4. Antigen #5, the supernate from trypsin digestion and 10% TCA precipitation, failed to show any hemagglutinating activity, indicating either loss of adsorptive property, or lack of antibody in the rabbit anti-SR-serum to this fraction, since this fraction showed skin reactivity in one SR sensitive subject and not in another.

A question raised is whether this decrease in titer shown in the table is due to free, eluted antigen competing with fixed antigen for the antibody. If eluted antigen was responsible, it would be possible to wash away the eluted antigen, and if enough antigen remained on the cells, they should again show agglutination to the original titer. So, the various tanned RBC-antigen preparations were allowed to incubate for 36 hours in saline suspensions. These were then mixed with serial serum dilutions. (These dilutions were aliquots of the master dilutions prepared 40 hours previously.) The titrations at 2 hours yielded titers identical to those shown in Table I for 36 hours. However, when aliquots of these cell suspensions held for 36 hours were washed and resuspended in fresh saline, and used in hemagglutination tests as before, the titer noted at the end of 2 hours' incubation was identical with the 2-hour titer recorded in Table I. Upon shaking these latter titrations and allowing a 36-hour incubation period, the titer again showed a decrease. These data indicate that the elution of antigen from the tanned-antigen-treated RBC is not a function of the contact of adsorbed antigen with antibody, but can occur in the absence of antibody.

To further prove the presence of eluted antigens in the supernates after 36 hrs incubation, the supernates were recovered and injected into the skin of a short ragweed sensitive individual whose skin was reactive to all 5 antigenic components in soluble form. Injection of 0.05 ml of each supernate showed immediate wheal and erythema reactions in 15 minutes. The intensity of these reactions was recorded, but since the subject had a greater or lesser sensitivity to each of the antigenic components, quantitation was impossible. However, the reactions did indicate that antigen eluted from each of the RBC preparations, and could be detected in the supernatant fluids.

Since the maximum hemagglutination titer is again seen after washing the cells free of eluted antigen, it is obvious that tanned RBC adsorb more antigen than is necessary to show hemagglutination.

Summary. (1) Pollen antigens (short ragweed) elute slowly from the surface of tanned sheep erythrocytes. (2) This elution does not apparently affect the titer obtained over a 2 hr incubation period. (3) Some antigenic fractions fix more permanently than others. (4) The elution is not a function of contact of the RBC-antigen with its specific antibody. (5) This method may possibly be used to separate various antigenic components in the search for antigens with fewer specificities.

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Effect of Combined Thyroxin-Cortisone-Growth Hormone Therapy on Hematopoiesis in Hypophysectomized Rats.* (23397)

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It has been well established that an anemia follows hypophysectomy. The role played by thyroid and adrenal glands in the etiology of this anemia has been the subject of several investigations. Combined thyroidectomy and adrenalectomy induced changes in the peripheral blood similar to those following hypophysectomy(1). In addition, combined thyroxin-cortisone therapy in hypophysectomized rats prevented occurrence of an anemia(2). Van Dyke, *et al.*(3), however, found that anemia following hypophysectomy was more severe than that following combined thyroidectomy, adrenalectomy and gonadectomy and proposed that the "erythropoietic hormone" from the pituitary was the explanation for this finding. This prompted a reinvestigation of this problem by the present authors (4); whereas the peripheral anemias in hypophysectomized and thyroidectomized-adrenalectomized rats were similar, the marrows were not identical. In addition, although we found (5) combined thyroxin-cortisone therapy would eliminate post-hypophysectomy anemia, the bone marrow was severely hypoplastic. When the whole picture was analyzed it was apparent that the pituitary gland was in some way playing a role in hematopoiesis exclusive of its effects on thyroid and adrenal glands.

The present investigation was performed to determine whether growth hormone could be this additional pituitary factor. Although several studies(6-11) have shown that growth hormone therapy has no effect on post-hypophysectomy anemia, both Fruhman and Gordon(10) and Meineke and Crafts(11) reported that growth hormone increased the number of erythroid elements in bone marrow. It seemed possible that a lack of growth hormone was involved in post-hypophysectomy anemia in addition to the hypothyroidism and hypoadrenocorticalism which inevitably follow removal of the pituitary gland. Since we have postulated that post-hypophysectomy anemia is a reflection of decreased oxygen need(12), oxygen consumption was also measured and correlated with blood and marrow findings.

Materials and methods. Female rats, 3 to 4 months of age, of the Sprague-Dawley strain were used and were fed standard Purina chow *ad lib.* supplemented once a week with lettuce. Completeness of hypophysectomy was checked at autopsy by examining the organ site with a dissecting microscope. Blood samples were obtained via heart puncture with the rats under ether anesthesia. The blood was transferred to heparinized vials. Erythrocyte and white cell counts were done in duplicate using U.S. Certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made in a Klett-Summerson photoelectric colorimeter. Hematocrit determinations were made with Van Allen hematocrit tubes (no diluent) and spun for one hour at 2,000 rpm. Blood volume measurements were made by the Evans Blue dye method as described by Metcoff and Favour (13). Total number of nucleated cells/mm^3 of marrow and actual number of myeloid and erythroid elements/mm^3 of marrow were determined by a method already published(11). The method used for determining oxygen consumption has been described(12).

Procedure and results. The rats in this experiment were divided into 3 groups: (1) normal controls, (2) hypophysectomized—no treatment, and (3) hypophysectomized for 4 months followed by daily injections of 0.005 mg thyroxin, 0.6 mg cortisone acetate[†] and

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[†] Cortisone Acetate generously supplied by Merck and Co., Inc.

TABLE I. Peripheral Blood Picture in 3 Groups of Adult Female Rats: (1) Normal Controls, (2) Hypophysectomized for 7 Months, and (3) Hypophysectomized for 4 Months Followed by Daily Injections of 0.005 mg Thyroxin, 0.6 mg Cortisone Acetate, and Gradually Increasing Amounts (0.2-0.8 mg) of Growth Hormone for 3 Months. ± = stand. error.

	Normal controls	Hyp., no treatment		Hyp., treated	
	8 rats	7 rats	% of normal	11 rats	% of normal
Body wt, g	293 ± 7.97	187 ± 8.28	64	302 ± 8.59	103
Erythrocytes, millions/mm^3	8.27 ± .17	7.71 ± .21	93	9.22 ± .19	111
Hematocrit, %	45.6 ± .7	38.1 ± .7	84	47.0 ± .4	103
Hemoglobin, g/100 cc	15.5 ± .2	12.6 ± .2	81	16.0 ± .1	103
Reticulocytes, %	1.1 ± .1	.9 ± .2	82	1.3 ± .2	118
Mean corpuscular vol, μ^3	55.1	49.4	90	51.0	93
" " hemoglobin, $\mu\mu$g	18.7	16.3	87	17.3	93
" " " conc., %	34.0	33.0	97	34.0	100
Total cellular vol, cc/100 g body wt	2.33 ± .06	1.73 ± .04	74	2.39 ± .08	103
Total white cell count, thousands/mm^3	10.3 ± .6	12.9 ± 1.0	125	12.6 ± .4	122

increasing amounts (0.2 mg increment every 10 days until 0.8 mg was being given) of growth hormone‡ for 3 months. The results are divided into 3 categories, *i.e.*, the effect (1) on peripheral blood picture (2) bone marrow, and (3) oxygen consumption.

Results of the peripheral blood studies are tabulated in Table I. Hypophysectomy induced the usual anemia which was, because of hemoconcentration, more severe than the peripheral blood picture indicated; total cellular volume/100 g body weight exhibited a 26% decrease. This anemia was accompanied by a slight increase in total white cell count and a lowered body weight. The 3 hormone therapy completely repaired the anemia with the total erythrocyte, hematocrit, hemoglobin, total cellular volume, body weight and total white cell values all at normal or slightly elevated levels. There were no significant differences found in the reticulocyte counts although there was a tendency for them to be lower after hypophysectomy and slightly elevated after treatment.

Histologic study of bone marrows (Fig. 1, 2, 3) showed that they were hypoplastic following hypophysectomy and indistinguishable from normal after hormone therapy. The data obtained from bone marrow counts are presented in Table II. In agreement with the histologic picture, there was a reduction in total number of nucleated cells/mm^3 of marrow tissue following hypophysectomy, and an elevation to normal following the 3 hormone therapy. The total nucleated cell count following hypophysectomy was not statistically different from normal; the trend indicated, however, is consistent with our past experience and the changes noted are believed to reflect biologically significant alterations in the marrow. Hypophysectomy induced a de-

TABLE II. Bone Marrow Picture in 3 Groups of Adult Female Rats: (1) Normal Controls, (2) Hypophysectomized for 7 Months, and (3) Hypophysectomized for 4 Months Followed by Daily Injections of 0.005 mg Thyroxin, 0.6 mg Cortisone Acetate, and Gradually Increasing Amounts (0.2-0.8 mg) of Growth Hormone for 3 Months. ± = stand. error.

	Normal controls	Hyp., no treatment		Hyp., treated	
	6 rats	6 rats	% of normal	10 rats	% of normal
Total nucleated cells, millions/mm^3 of marrow tissue	1.748 ± .06	1.597 ± .13	91	1.724 ± .06	99
Total erythroid elements, thousands/mm^3 of marrow tissue	866.4 ±107.4	633.8 ±54.8	73	709.1 ±49.0	82
Total myeloid elements, thousands/mm^3 of marrow tissue	698.4 ±101.4	724.6 ±59.4	104	784.4 ±44.9	112

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‡ Growth Hormone Lot R377237 and Somar Lot M208 generously provided by Armour Co.

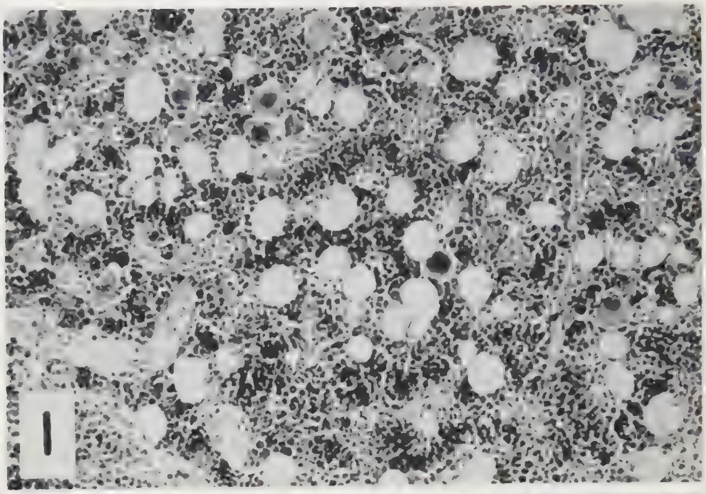

FIG. 1. Femur marrow from normal rat ×160. Note small amt of fat and dark islands of cells representing erythroid elements.

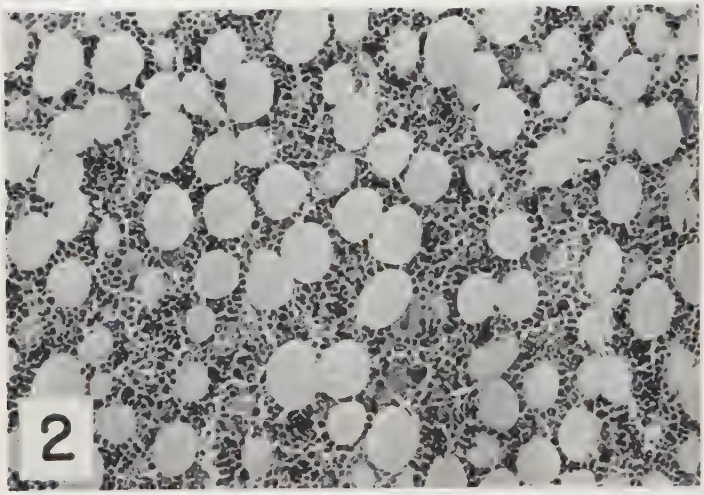

FIG. 2. Femur marrow from hypophysectomized rat 7 mo after surgery × 160. Note increase in amt of fat and that erythroid islands are small and indistinct.

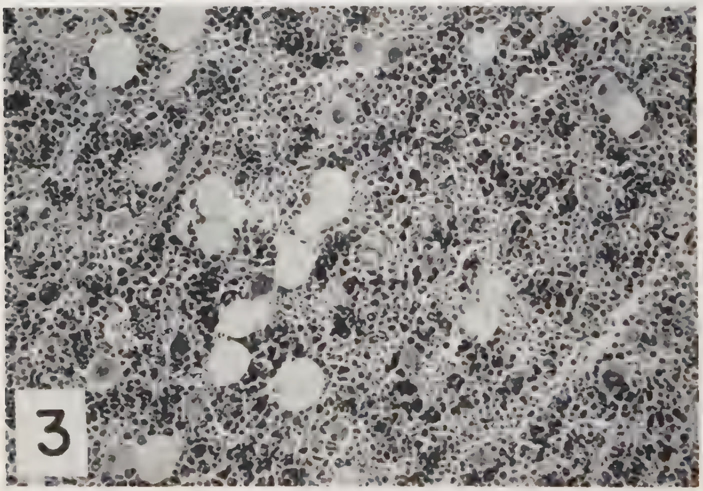

FIG. 3. Femur marrow from rat hypophysectomized for 4 mo followed by daily inj. of 0.005 mg thyroxin, 0.6 mg cortisone acetate, and gradually increasing amounts (0.2 to 0.8 mg) of growth hormone for 3 mo × 160. Note small amt of fat and normal appearance of erythroid islands.

TABLE III. Oxygen Consumption in 3 Groups of Adult Female Rats: (1) Normal Controls, (2) Hypophysectomized for 7 Months, and (3) Hypophysectomized for 4 Months Followed by Daily Injections of 0.005 mg Thyroxin, 0.6 mg Cortisone Acetate, and Gradually Increasing Amounts (0.2-0.8 mg) of Growth Hormone for 3 Months. ± = stand. error.

	Normal controls	Hyp., no treatment		Hyp., treated	
	8 rats	7 rats	% of normal	11 rats	% of normal
Oxygen consumption, l/m² body surface/hr	8.20 ± .24	6.98 ± .27	85	7.76 ± .25	95

crease in erythroid elements in the marrow to 73% of normal but induced no significant changes in number of myeloid elements. The 3 hormone treatment elevated myeloid elements to 12% above normal; the count for erythroid elements was 82% of normal.

Oxygen consumption by the 3 groups of rats studied is presented in Table III. Hypophysectomy induced a decrease in oxygen consumption which was returned to 95% of normal by the 3 hormone therapy.

Discussion. Previous work(3,4) has shown that blood and bone marrow pictures following combined thyroidectomy-adrenalectomy are not identical with those following hypophysectomy. This suggests that removal of some pituitary factor, in addition to those regulating the thyroid and adrenal cortex, is needed to explain the peripheral blood and bone marrow picture following hypophysectomy.

Insofar as the peripheral erythrocyte picture is concerned, the results in the present experiment show that the anemia is completely repaired by a combined thyroxin-cortisone-growth hormone therapy. This repair to the peripheral erythrocyte number was not different from that obtained with thyroxin-cortisone treatment alone(2,5) but the bone marrow studies demonstrate differences worthy of some discussion.

In degree of cellularity the marrows of the animals treated with all 3 hormones were indistinguishable from normal, which is in contrast to the hypoplastic marrow of thyroxin-cortisone treated rats(5) or the hyperplastic marrow of rats treated with growth hormone(11). This histological picture is paralleled by total nucleated cell counts of the marrows; the number of cells is normal in rats treated with the 3 hormones, 69% of normal in thyroxin-cortisone treated animals (5) and 128% of normal in those treated with growth hormone(11). These results, however, do not indicate whether changes were in the erythroid or myeloid series of cells.

Treatment with thyroxin-cortisone alone (5) induced a marked depression of the total number of *myeloid* elements in the marrow (52% of normal) which was reflected in the peripheral blood by a reduced total white cell count (58% of normal). Growth hormone alone, in contrast, resulted in elevated counts of both the marrow myeloid elements and the peripheral white cell count(11). Combining all 3 hormones, as shown in the present experiment, prevents these changes; total number of marrow myeloid elements and peripheral white cells were closer to normal levels. It should be noted that changes in marrow myeloid elements were reflected in similar changes in numbers of peripheral white cells. (The part lymphocytes are playing in the total white cell changes has not been studied other than to note that, in general, there is an increase in marrow lymphocytes after hypophysectomy which is returned to near normal levels by all therapies mentioned.)

Changes in marrow *erythroid* elements are more difficult to interpret because changes in marrow and peripheral blood do not always parallel one another; there is a difference between production of erythrocytes in the marrow and delivery of these cells to the peripheral blood. Hypophysectomy induced a decrease in erythroid elements to 73% of normal which was reflected in a peripheral reduction of total red cell volume/100 g body weight to 74% of normal. Previous work(5) has shown that combined thyroxin-cortisone therapy in hypophysectomized rats maintained a normal peripheral erythrocyte level but did so with a marrow which was gradually being exhausted; number of erythroid elements was 83% of normal. Growth hormone(11), in contrast, increased the marrow erythroid elements to a level 49% above normal; in spite of this increased production, these cells were not delivered to the peripheral blood—the anemia persisted. Combining all 3 hormones induced changes in marrow erythroid elements and in total cellular volume of peripheral erythrocytes/100 g of body weight similar to those obtained with combined thyroxin and cortisone therapy.

This lack of an effect of growth hormone on the erythroid elements when combined with thyroxin and cortisone is difficult to explain when it has been shown that growth hormone has a profound effect on these elements when given alone(10,11). We feel that this finding is a matter of rate of production and release of erythroid elements and is a result of imbalance in hormone dosage. This conclusion agrees with the work of Fruhman and Gordon(10) who reported that growth hormone and cortisone were antagonistic to one another in their effects on development of both erythrocytes and white cells, and that the number of erythroid elements in bone marrow of rats treated with cortisone and growth hormone was intermediate between rats treated with either hormone alone. Evidence in the present experiment shows that growth hormone, when given in conjunction with thyroxin and cortisone, is not completely inactive; it has prevented the marked loss of myeloid elements and peripheral white blood cells seen in thyroxin-cortisone treated hypophysectomized rats to such an extent that the number of cells/mm^3 of marrow is normal as is the peripheral white cell number.

Is the loss of growth hormone a factor, in addition to thyrotropic and adrenocorticotropic hormones, which is responsible for hematological changes found in hypophysectomized rats? It seems clear that growth hormone has a stimulating effect on both myeloid and erythroid elements in bone marrow when given alone(10,11), although this may be a general effect of growth hormone injections rather than any specific one on marrow cells. The present experiment, however, allows the conclusion that growth hormone has an effect on myeloid elements, counteracting effects of thyroxin-cortisone injections; if it had an effect on erythropoiesis in the present experiment, it was masked by the reverse effects of cortisone.

Previous work(12) has shown that oxygen consumption is returned to normal levels by thyroxin-cortisone therapy but is not affected by growth hormone alone. It was postulated that decreased oxygen need is a causative factor in the anemia following hypophysectomy. The present experiment provides another example of repair of oxygen consumption with a parallel alleviation of the peripheral anemia.

Summary. Previous work has shown that a combined thyroxin-cortisone therapy in hypophysectomized rats will eliminate the anemia and induce a marked decrease in peripheral white cells; this was accompanied by severe hypoplasia of the bone marrow. It has also been shown that growth hormone increases peripheral white cell count in hypophysectomized rats but has no effect on peripheral anemia; the bone marrow was hyperplastic. Adding growth hormone to the thyroxin-cortisone therapy resulted in repair of post-hypophysectomy anemia, in normal histological appearance of bone marrow, in normal total number of cells/mm^3 of marrow tissue, and in values for myeloid elements/mm^3 of marrow and peripheral white cells slightly above normal. The values for erythroid elements/mm^3 of marrow averaged 82% of normal. These hematological findings were accompanied by a normal oxygen consumption.

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Effect of *Candida Guilliermondi* Polysaccharide on Transplantable Mouse Sarcoma 37. (23398)

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Previous experiments conducted in this laboratory(1) indicated that several species of *Candida* and *Saccharomyces* exerted a lytic action on Sarcoma 37 ascites and Ehrlich ascites cells *in vitro,* either when incubated together in isotonic glucose solution, or when grown simultaneously in tissue culture; though normal mouse tissues, similarly treated, remained unharmed. These results confirmed and extended those of several earlier workers, already reviewed(1). When we injected mice bearing solid transplantable Sarcoma 37 with living and killed *Candida* species by various routes, sloughing of tumors occurred in varying percentages (approximately 90% with living *C. Guilliermondi* injected intravenously(2,3). The fate of the organisms in tumor and host tissues of intravenously injected mice was followed, and the selectively destructive action of the organisms, administered in non-toxic but tumor-necrotizing doses, was confirmed(4). Heat- and chemically-killed cultures were active, though to a lesser degree(3) and it was therefore decided to test whether some effective agent could be isolated by chemical means from killed or lyophilized cultures.

Our first attempt was to separate a polysaccharide fraction by the procedure of Pillemer *et al.*(5). Since *Candida Guilliermondi* was the most suitable organism for use in the living state because of its high tumor-necrotizing properties with respect to Sarcoma 37, and its nonpathogenicity to mice, cultures of this organism were chosen as our starting material.

Methods. The *Candida* cells were grown in 12 liter flasks on glucose-peptone medium (1% peptone, 2% glucose) and were aerated at room temperature. After 3 days these cultures were centrifuged in a Westphalia apparatus. The yield was, on the average, 300 g of wet yeast cells. These cells were treated according to Pillemer's method for extraction of zymosan, as follows:

Three hundred grams of yeast cells were suspended in 1200 ml of 0.5 *M* sodium monohydrophosphate solution and boiled for 3 hours. To this solution was added 0.17 g of trypsin (Wilson 1:250) on the 1st, 3rd, 6th, and 10th day of incubation at 37°C. Two-tenths ml of toluene was also added on the 1st, 5th, 8th and 11th days. Incubation was continued for 16 days at the same temperature, with occasional shaking, and the pH of the liquor of incubation was adjusted daily to 7.8-8.0 by careful addition of 2 *N* NaOH. The liquor was then centrifuged, the supernate discarded, and the precipitate stirred with 1200 ml of water at room temperature for 1 hour and again separated by centrifugation. The resulting precipitate was suspended with vigorous stirring in 1200 ml of water, maintained at 95-100°C for 1 hour, and recentrifuged while hot. This procedure was repeated twice more. No trace of glucose was found in the final supernate upon testing with Fehling's solution. The precipitate was stirred vigorously with 2400 ml of absolute ethyl alcohol for 1 hour, centrifuged, and the precipitate treated with 1200 ml of absolute alcohol as before. The residue was refluxed for 3 hours with 150 ml of absolute ethyl alcohol, centrifuged and dried *in vacuo,* with a final yield of approximately 4 g of dried material. Fehling's test, following treatment with HCl, gave a positive reaction for carbohydrate.

Results. Three groups of Swiss mice (100 each) were implanted subcutaneously with Sarcoma 37 and the tumors were allowed to grow for 1 week, or until they averaged 1 cm in diameter. One group was treated with the polysaccharide derived from *C. Guilliermondi;* the second group received Fleischman's zymosan in the same dosage; and the third group received no treatment. Since intravenous injection resulted in an undesirably high mortality rate, administration was made routinely by the intraperitoneal route. Each mouse received 2 doses of 0.4 mg each, 2 days apart. These injections were well tolerated, and there was no local tissue reaction at the injection site.

Twenty days after the initial injection, complete regression of tumors had occurred in 62% of the mice treated with the *C. Guilliermondi* product, in 67% of mice treated with commercial zymosan, and in 6% of the controls. The remaining tumors in each group continued to grow until the death of the hosts (mean survival time, 28 days). Mice whose tumors had regressed were observed for at least one month subsequent to the death of the last control mouse. Regrowth of tumors did not occur. While these experiments were in progress Bradner, *et al.*(6), reported similar regression rates for Sarcoma 180 treated with zymosan.

It will be seen that the polysaccharide extracted from *C. Guilliermondi* at the dose levels employed, and intraperitoneally administered, was not as effective as were the living cells used in previous experiments, which brought about regression of tumors in 80-90% of the treated hosts; however, even living cultures, when injected by the intraperitoneal route, were not as effective as when intravenously administered.

Histological preparations of non-growing tumors at 24 hours, 48 hours, and 72 hours post-injection, revealed progressive destruction of tissue, involving dissociation of cells, destruction of cell nuclei, and extrusion of chromatin filaments, similar to that observed following treatment with bacterial polysaccharides. No hemorrhage was noted either macroscopically or microscopically. At 72 hours the tumor tissue was riddled with necrotic islands composed of enucleated cells and granular debris. There was no mitotic activity and the peripheral stroma was devoid of tumor cells.

Thus far, no effect has been observed on Sarcoma 37 ascites tumors when the host mice were injected intraperitoneally with either of these polysaccharides, and no prolongation of life was achieved. Previous trials had shown that living *C. Guilliermondi,* injected intravenously, caused spontaneous mammary tumors of C3H and Swiss mice to remain static for considerable periods of time, with occasional slight reduction in size, but no effect was observed on such tumors in mice injected intraperitoneally with these yeast polysaccharides.

Summary. Complete regression of subcutaneously implanted Sarcoma 37 occurred in 62% of host mice treated with a polysaccharide derived from *Candida Guilliermondi,* in 67% of those treated with Fleischman's zymosan, and in 6% of the controls. No toxic symptoms developed following intraperitoneal injection of either of these polysaccharides, and no hemorrhagic reaction was noted in tumor tissues. Neither substance had any effect on the ascites form of Sarcoma 37 *in vivo.*

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Antigenicity of Zymosan. (23399)

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Pillemer *et al.*(1) have demonstrated that zymosan, the soluble carbohydrate residue from yeast cells, combines with the properdin of fresh serum at 17°C to form an insoluble complex thereby removing the capacity of the normal serum to destroy bacteria, neutralize viruses, and lyse red cells.

Previously(2), it was demonstrated that the serum of rabbits and guinea pigs which had been injected with zymosan incorporated in adjuvants showed an increased bactericidal activity for *Escherichia coli* B. Skin testing of these animals with zymosan resulted in an Arthus reaction thereby suggesting the possible antigenicity of zymosan.

Additional experiments have been carried out in rabbits injected with zymosan plus adjuvants. It has been demonstrated by a quantitative agglutination technic that heat stable antibodies to zymosan are formed and that a concomitant increase in bactericidal activity for *E. coli* B can be absorbed by *E. coli* B cells.

Methods. Zymosan,* suspended in saline instead of egg-white, was prepared for injection by incorporation in Freund adjuvants as previously described(2). The presence of egg-white was found to be unnecessary.

Young adult male rabbits were used. Each of 6 rabbits received a subcutaneous injection of 8 mg of zymosan in adjuvants† followed by a 16 mg injection one week later. Five rabbits received no injections and were regarded as normal controls. Three rabbits received 2 injections of adjuvants without zymosan to test the effect of adjuvants alone. Two additional groups of 3 rabbits each were injected with zymosan alone, one group receiving 2 I.V. injections a week for 3 weeks and the other group 2 intracutaneous injections a week for 3 weeks. Each rabbit received a total of 12 mg of zymosan. All animals were bled from the heart before the injections began and periodically up to 100 days after the last injection. Sera from the rabbits within each group were pooled at each bleeding and placed in sealed tubes in a dry ice chest.

Bactericidal tests were carried out with *E. coli* B as previously described(2). Antigen for quantitative agglutination test was prepared by thoroughly grinding 0.1 g of zymosan in an agate mortar and slowly suspending it, with grinding, in 20 ml of sterile saline. The suspension was heated in a boiling water bath for 1½ hours. After centrifugation for 30 minutes at 1770 g, the precipitate was resuspended in 20 ml of sterile saline according to the method of Pillemer *et al.*(3). This suspension was stored in the refrigerator and usually used within the next 2 days. To 1 ml of serum to be tested, 1 ml of zymosan suspension (5 mg) was added. The mixture was shaken and placed in a 16.5°C water bath for 2 hours; during this time the tubes were well mixed every 10-15 minutes. The flocculant precipitate was centrifuged at 1550 g for 20 minutes and washed twice with 2 ml of ice-cold saline as described by Kabat and Mayer (4). Five mg of zymosan was found to be sufficient as further addition of zymosan to the supernatant failed to precipitate additional antibody.

All centrifugations and washings were conducted at 2-5°C in a refrigerated room. The washed precipitate was suspended in 4 ml of 0.25 N acetic acid and the supernatant clarified by centrifugation. The supernatant was read in the Beckman DU spectrophotometer at 277 mμ(5), with 0.25 N acetic acid in the reference cell. A zymosan blank was carried through the procedure without added serum. Optical densities of supernatants were corrected for the optical density of the blank which never read more than .005. The results were calculated in μg N/ml from E =

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* Standard Brands, Inc.

† Adjuvants are Bayol F (Esso Standard Co.), Falba (Pfaltz and Bauer, Inc.), and killed tubercle bacilli strain H37Ra.

$\frac{\text{O.D.}}{\mu\text{g N in sample}}$ where E, the extinction coefficient, is 0.0099 as determined by McDuffie and Kabat(5) for rabbit antibodies.

E. coli B was grown on nutrient agar, thoroughly washed with distilled water, centrifuged, suspended in 0.4% formalin and incubated until no longer viable as determined by plate count. Before use, the formalin was removed by centrifugation and the cells suspended in saline to a concentration of 2.2 x 10^9/ml. For absorption, 1 ml of cell suspension was added to 1 ml of serum and incubated at 16.5°C for 2 hours with shaking at 10-15 minute intervals. The suspensions were centrifuged in the refrigerated room and the supernatants were tested for remaining bactericidal and hemolytic activity.

Results. Fig. 1 indicates the rise and fall of the rabbit antizymosan serum titer. Zymosan plus adjuvants caused a delayed but sharp rise followed by a slow decline. The intravenously injected rabbits seemed to have been little affected by the injections. The rabbits given zymosan intracutaneously showed a definite though small increase in serum titer during the first 4 weeks. The titer of rabbits injected with adjuvants alone rose to about twice its original level and remained there throughout the experiment. Each point in Fig. 1 is the average result of 3 or more determinations performed on separate days. Four sera were titrated 7 times each and the standard deviation was calculated from $S = \sqrt{\frac{\Sigma x^2}{n-1}}$ with the following results in µg N/ml: 231.6, S = 2.43; 237.8, S = 3.20; 81.9, S = 3.45; 20.2, S = .70.

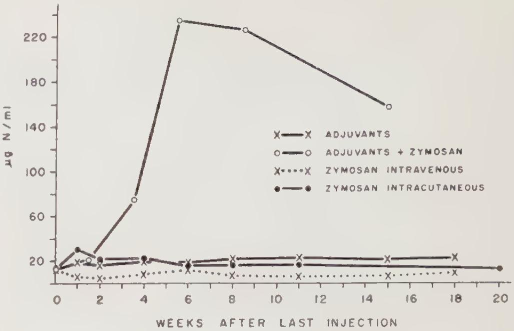

FIG. 1. Comparison of amt of nitrogen precipitated by zymosan from sera of rabbits immunized according to the various regimens.

Antibody nitrogen determinations made on immune sera, heated to 56°C for 30 minutes, were not found to be significantly different from the determinations made with unheated sera. It is therefore concluded that the antibodies are heat stable.

The bactericidal activity of the sera of rabbits bled 3½, 5½, and 8½ weeks after immunization with zymosan plus adjuvants was compared with the activity of sera of normal rabbits bled at the same time. The sera were absorbed with *E. coli* B at 16.5°C for 2 hours and then again tested for bactericidal activity. Table I records the results on the 5½ week sera. The 3½ and 8½ week sera gave similar results. The immune sera had greater bactericidal activity than normal sera, and after absorption with *E. coli* B neither exhibited bactericidal activity. Wardlaw and Pillemer(6) have demonstrated that lysis of bacteria requires all 4 components of com-

TABLE I. Bactericidal Activity. Viable counts of *E. coli* B/ml after incubation in sera of rabbits immunized according to the various regimens.

Sera	Final % conc. in test	Hr incubation 1	2	3	4
Adjuvants + zymosan	.75	3	0	0	0
” ” ” “absorbed”*	1	118	340	TM†	TM†
Normal	1	79	47	225	”
” “absorbed”*	1	101	350	TM	”
Adjuvants	1	86	45	180	”
Zymosan intrav.	1	71	44	200	”
” intracut.	1	49	32	140	”

* Sera were absorbed with 2.2 × 10^9 *E. coli* B/ml.
† TM = Too many colonies to count.
Plate count of *E. coli* B at start of exp. 100 ± 5/ml.
Each plate count is the avg of 3 exp. set up on different days.

plement. Therefore, after absorption with *E. coli* B the sera were titrated for hemolytic activity in a standard test with sensitized sheep cells. Less than 15% of their original activity was lost so that the decrease in bactericidal activity could not be attributed to a loss of complement. Table I also records the bactericidal activity of normal sera, of the sera of rabbits injected with adjuvants alone, and with zymosan alone injected intravenously or intracutaneously. These sera were obtained 2 weeks after the last injection. It can be seen that there is no evidence of increased bactericidal activity nor was any increase found in later bleedings. Each viable count in Table I represents the average of 3 experiments.

Discussion. Previously it was demonstrated that animals injected with zymosan incorporated in adjuvants would respond to an intracutaneous injection of zymosan with an Arthus phenomenon(2). Further evidence of the antigenicity of zymosan is demonstrated by the rise and fall of nitrogen content of zymosan antibodies in sera of rabbits immunized with zymosan plus adjuvants. These antibodies were shown to be heat stable.

The small increase in antibody nitrogen evident at the first week and continuing at a reduced level for the next 3-4 weeks in sera of rabbits injected intracutaneously with zymosan might indicate that, had much larger amounts of zymosan been injected, it is possible that high titered sera could have been produced without the aid of adjuvants. The intravenously injected rabbits too may not have received sufficient antigen.

The small increase in precipitable nitrogen in the sera of rabbits injected with adjuvants alone would indicate a substance in the adjuvants to which the rabbits responded and which was precipitated by the zymosan. Further investigation(7) revealed that rabbits injected with Bayol F or Falba, either alone or in combination, failed to give increased precipitates with zymosan. However, the killed tubercle bacilli, either alone or in combination with Bayol F or Falba, was found to be the cause of the increased titer represented in Fig. 1. It may be that the tubercle bacilli contain an antigenic grouping in common with zymosan.

Further it should be noted that rabbits immunized with zymosan plus adjuvants plus egg-white(2) when tested by quantitative agglutination with zymosan gave results of the same order as those given by rabbits without the addition of egg-white(7). Since a high titer of anti-egg-white antibodies was produced in these rabbits, and since the zymosan antizymosan titer was not increased, it would seem that in this case at least, zymosan did not precipitate the nonspecific gamma globulin.

It has been demonstrated that either zymosan or *E. coli* B is capable of absorbing bactericidal activity of normal as well as of zymosan immune sera(2). In addition, injections of zymosan were capable of increasing bactericidal activity of rabbit sera for *E. coli* B. It would seem, therefore, that *E. coli* B and zymosan may be related antigenically through spatial arrangements or specific configurations.

It is likely that zymosan contains a number of antigens due to the fact that it is a relatively crude commercial preparation derived from the cell walls of yeast. It is suggested that the increased bactericidal activity to *E. coli B* is a response to one of the antigenic groupings on the zymosan molecule, and that the amount of bactericidal antibody is a small part of the total antibody precipitated from the immune sera by zymosan.

Further work is planned to define more clearly the relationship between bactericidal and anti-zymosan properties of the sera of rabbits injected with zymosan.

Summary. Heat stable antibodies to zymosan were produced in rabbits and measured by quantitative agglutination. The increased bactericidal activity for *Escherichia coli* B resulting from injections of zymosan could be absorbed by *E. coli* B. Some aspects of the antigenicity of zymosan are discussed.

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Effect of Partial Hepatectomy on Liver and Kidney Coenzyme A Content in the Rat.* (23400)

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The rat liver regenerates rapidly after surgical extirpation of as much as two-thirds of the liver mass. During the regenerative process protein content and organ weight are restored to the original levels within a period of from 14 to 21 days after partial hepatectomy(1,2). Protein synthesis proceeds most rapidly in the first 4 to 8 days and affords the opportunity for study of protein anabolic processes in liver. The investigation described below was undertaken in the effort to evaluate the role of coenzyme A in this process. Acetyl coenzyme A is important in systems of carbohydrate and fat metabolism through mechanisms of utilization of 2 carbon fragments(3,4,5,6,7). It is to be presumed that similar mechanisms are involved in systems of protein metabolism. In this investigation the concentrations of coenzyme A in liver and kidney of rats were studied during the period of increased protein synthesis induced by subtotal hepatectomy.

Procedure. Male Sprague-Dawley rats weighing 95 to 135 g were divided into 4 groups. A subtotal hepatectomy was performed on 44 rats in Group I by excision of median and left lateral liver lobes *in toto* leaving the posterior and caudate lobes intact (major hepatectomy). The amount of liver removed was approximately 66% of total wet liver weight, a value corroborated by performing the identical operation on 10 additional animals which were immediately sacrificed and the excised and remaining liver mass determined. Rats in this group were sacrificed at intervals up to 14 days following major hepatectomy. Group II consisted of 10 rats which were subjected to excision of the left lobule of the median lobe of the liver (minor hepatectomy). Minor hepatectomy thus performed removed approximately 8.5% of total liver, or about ⅛th as much liver tissue as was removed at major hepatectomy (Group I). Animals in Group II were sacrificed 24 hours post-operatively and served as a control in evaluation of surgical stress and trauma to the liver on hepatic coenzyme A concentrations. Group III included 10 rats which were subjected to major hepatectomy and sacrificed 24 hours later, in order to determine the effect of early liver regeneration on kidney coenzyme A concentrations. Group IV consisted of 10 normal rats which were sacrificed and kidney coenzyme A concentrations determined. This group thus served as a control for Group III.

All animals were fed a stock diet (Purina Laboratory Chow) *ad libitum*. The daily food consumed by each rat in Group I was measured for one week prior to surgery and throughout the post-operative period daily intake of calcium pantothenate was estimated from amount of food consumed. Hepatectomized rats in Groups I, II and III received 15,000 units of procaine penicillin, intramuscularly, immediately following surgery. Body weights were measured at time of hepatectomy and at sacrifice. At the conclusion of the experimental periods, animals were decapitated. The remaining hepatic tissue and both kidneys were removed and weighed.

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† Fellow of National Foundation for Infantile Paralysis.

Coenzyme A and protein analyses were performed on liver tissue removed at operation and at sacrifice of animals in Groups I and II and on kidney tissue obtained at the time of sacrifice of rats in Groups III and IV. Tissues were immediately blotted, weighed, and homogenized in 9 volumes of distilled water in a Potter-Elvehjem apparatus at 0°C. An aliquot of each homogenate was placed in boiling water for 5 minutes, then cooled in crushed ice. The cooled homogenates were centrifuged to remove precipitated material and an aliquot of supernatant solution analyzed for coenzyme A. Liver tissue obtained at hepatectomy was prepared in the above manner and the supernatant solutions were stored at –10°C until respective animals were sacrificed. Thus, coenzyme A assays were performed simultaneously on tissue removed at surgery and at sacrifice. The remainder of each homogenate was frozen and set aside for protein determination by the copper-Folin method of Lowry(8).

Coenzyme A analyses were performed by the arsenolysis method(9) with minor modification. Dilithium acetyl phosphate was prepared by the reaction of isopropenylacetate with phosphoric acid and purified by 3 precipitations with absolute ethanol(10). Crude phosphotransacetylase was obtained from Dowex-1 treated *Cl. kluyverii* cells.‡ Standard coenzyme A (Sigma Chemical Co.) providing 300 Lipmann units per mg was diluted with distilled water to obtain 10 units per ml. Analyses were performed on 0.3 ml of each tissue supernate and compared with the standard solution containing 3 units of coenzyme A. All solutions were read in the Coleman spectrophotometer at a wave length of 540 mμ. Coenzyme A concentrations were expressed as units per g wet tissue and as units per 100 mg tissue protein. Tissue protein concentration was considered a more suitable standard of reference than desoxypentosenucleic acid (DNA) since both increased and decreased hepatic DNA concentrations have been noted during early phases of liver regeneration(11).

Preliminary studies on 5 animals revealed coenzyme A concentrations to vary less than 8% between the median and posterior liver lobes, a difference which was not statistically significant ($p > 0.1$). Thus, the possibility that inherent differences in coenzyme A concentration between liver lobes could account for the changes found was excluded.

Results. The majority of animals subjected to surgical procedures demonstrated weight loss during the first 2 to 3 post-operative days (Table I). A portion of this loss was attributed to removal of liver tissue at surgery, but the major loss of body mass was due to decreased food intake. During the first 24 hours following surgery, calcium pantothenate intake varied from 0.033 to 0.111 mg, with a mean of 0.090 ± 0.007 mg. During the next 24 hours, mean calcium pantothenate intake was 0.164 ± 0.016 mg, with a minimum of 0.135 mg. The latter values far exceeded minimal daily requirements reported for rats(12,13).

Rates of liver regeneration following major hepatectomy followed fairly closely those previously reported(2,14). Kidney weights were unaffected by major hepatectomy. Liver coenzyme A concentrations in Groups I and II are summarized in Table I. During the first 3 days following major hepatectomy (Group I), coenzyme A concentrations were significantly diminished in regenerating liver tissue when compared to levels present in liver tissue removed at time of partial hepatectomy. Decreases were apparent when coenzyme A concentration was based upon either wet tissue weight or upon tissue protein concentration. The lowest coenzyme A concentrations were observed 24 hours after surgery. By the fifth post-operative day, coenzyme A concentrations in regenerating livers were only slightly less than in their surgically removed counterparts, the differences attaining a low degree of significance. On the 8th and 14th days, coenzyme A levels in surgically removed and regenerating liver tissue were not significantly different. The slight decrease in hepatic coenzyme A concentration noted 24 hours after minor hepatectomy (Group II)

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‡ Extraction and Dowex-1 treatment of the dried cells were carried out by the Sigma Chemical Co., St. Louis, Mo.

TABLE I. Liver Coenzyme A Content* in Partially Hepatectomized Rats.

Days regeneration	No. rats	Mean body wt (g) Initial	Mean body wt (g) Final	Units/g wet liver Initial‡	Units/g wet liver Final§	% change	Significance of change (p value)	Units/100 mg tissue protein Initial	Units/100 mg tissue protein Final	% change	Significance of change (p value)
					Group I (major hepatectomy)						
1	10	120	115	142.2 ± 11.3†	99.4 ± 5.7	−30.0	<.001	86.8 ± 6.8	69.2 ± 4.3	−20.2	<.005
2	7	125	116	147.0 ± 10.2	106.5 ± 8.8	−27.6	<.02	86.5 ± 5.2	71.3 ± 5.5	−17.6	<.05
3	6	117	108	153.8 ± 5.2	118.3 ± 7.7	−23.1	<.005	93.7 ± 1.9	77.3 ± 3.8	−17.5	<.005
5	8	102	113	127.2 ± 9.1	112.5 ± 9.1	−11.6	>.05	73.2 ± 3.5	66.1 ± 4.6	− 9.7	>.05
8	8	110	137	140.5 ± 12.6	142.2 ± 8.2	+ 1.2	>.90	84.3 ± 6.1	87.3 ± 4.5	+3.6	>.60
14	5	114	116	129.8 ± 14.7	140.6 ±13.8	+ 8.3	>.10	78.8 ± 8.7	79.5 ± 8.5	+ .90	>.70
					Group II (minor hepatectomy)						
1	10	107	103	127.0 ± 6.5	113 ± 5.7	−11.0	<.02	75.8 ± 3.4	68.8 ± 2.9	− 9.2	<.005

* Expressed as Lipmann units. † S.E. of mean. ‡ At operation. § At necropsy.

was statistically significant. However, this decrease was only ⅓ (units per g wet tissue) to ½ (units per 100 mg of tissue protein) as great as that observed 24 hours after major hepatectomy. An analysis of variance in Groups I and II revealed the differences in response of hepatic coenzyme A levels to the 2 surgical procedures were highly significant ($p << 0.01$). The differences in coenzyme A concentrations of liver tissue removed at surgery in Group I series and Group II rats were not significant.

Kidney coenzyme A concentrations are presented in Table II. The mean coenzyme A concentration of kidney tissue 24 hours after major hepatectomy was slightly (16%) but significantly greater than that found in unoperated animals. Protein concentrations of the kidney and liver are presented in Tables II and III. Significant decreases in protein concentration were observed in regenerating livers of Group I during the first 3 post-operative days, as has been previously reported (15,16). Diminished hepatic protein concentration may result from an increase in hepatic fat and water content which has been observed early during liver regeneration(15, 16, 17) or from partial starvation(18,19). It should be noted, however, that no significant alterations in protein levels occurred in Group II livers nor in Group III kidneys.

Discussion. The results of these experiments clearly demonstrate a decrease in hepatic coenzyme A concentration during the first 3 to 5 days after partial hepatectomy. This period has been shown to be one in which liver regeneration proceeds most rapidly(2, 15). Factors which may result in decreased coenzyme A levels during this interval must be considered. It has been mentioned that an increase in fat and water contents of liver occur early in the regenerative period. The effects of fat infiltration and increased water content of hepatic tissue were minimized by expressing coenzyme A levels as units per 100 mg of tissue protein. Decreased coenzyme A concentrations remained significant when expressed in this manner. Therefore, it is unlikely that either fat or water content was of importance in lowering hepatic coenzyme A

TABLE II. Renal Coenzyme A and Protein Contents in Partially Hepatectomized and Non-Operated Rats.

	No. of rats	Body wt, g	Coenzyme A		Protein conc., mg/g wet tissue
			Units/g wet tissue	Units/100 mg protein	
Group III†	10	109	53.6 ± 1.4*	39.4 ± 1.1*	136 ± 2
" IV (control)	10	113	46.2 ± 2.3	33.9 ± 1.7	137 ± 2

* Difference from Group IV (control) significant, $p < .02$.

† Determinations 24 hr after two-thirds partial hepatectomy.

levels. A deficient intake of calcium pantothenate associated with partial starvation was noted in several animals on the first postoperative day only. Although an adequate intake of pantothenic acid is necessary to maintain normal tissue coenzyme A levels, it is improbable that starvation was a factor resulting in diminished hepatic coenzyme A concentrations in the present experiment. Ringler has shown that total starvation for 4 days had little effect upon liver coenzyme A levels in the rat(20).

Surgical stress and trauma to the liver may contribute to decreased hepatic coenzyme A concentrations since slight, but statistically significant, decreases in liver coenzyme A were observed after the stress of laparotomy and minor hepatectomy. However, these decreases were much less than those observed in the rapidly regenerating liver which remained after excision of two-thirds of the total liver. Therefore, stress and liver injury were not solely responsible for the marked reduction in liver coenzyme A concentration observed after major hepatectomy.

The lowered coenzyme A levels present in early liver regeneration may be associated with metabolic alterations which precede and accompany active tissue growth. Novikoff and Potter(11) demonstrated a marked lowering of certain hepatic Kreb cycle oxidases beginning as early as 15 hours and persisting 3 to 4 days after partial hepatectomy. This reduction in enzymes concerned with carbohydrate metabolism is paralleled closely both in degree and duration by the decreased coenzyme A concentrations present in rapidly regenerating liver tissue. In addition, markedly reduced coenzyme A levels have been demonstrated in actively growing induced rat liver tumors(21), paralleling an earlier observation that rat liver tumors oxidize oxaloacetic acid poorly(22). It becomes apparent that during rapid tissue growth and repair carbohydrate metabolism becomes subsidiary to protein synthesis, and that coenzyme A concentrations more closely parallel the former. If the tissue concentration of an enzyme is an index of its relative activity, the data suggest that coenzyme A may not be of major importance in the synthesis of tissue proteins.

TABLE III. Liver Protein Content in Partially Hepatectomized Rats.*

Days regeneration	Protein conc., mg/g wet liver		% change	Significance of change (p value)
	Initial	Final		
		Group I		
1	164 ± 2	146 ± 2	−11	<<.001
2	170 ± 7	149 ± 4	−12	<.02
3	164 ± 3	153 ± 5	− 7	<.05
5	173 ± 6	170 ± 5	− 2	>.90
8	165 ± 4	163 ± 3	− 1	>.90
14	164 ± 4	179 ± 4	9	>.10
		Group II		
1	167 ± 3	164 ± 2	− 2	>.30

* Determinations on same animals charted in Table I.

No adequate explanation for the observed rise in renal coenzyme A concentration following major hepatectomy is suggested by the data presented. It is entirely possible that the rise is compensatory to loss of a substantial portion of the total body coenzyme A via hepatectomy.

Conclusion. 1. Following partial hepatectomy in the rat, liver coenzyme A concentrations were found to be significantly reduced throughout the period of rapid liver repair. Neither fat nor water infiltration of liver, partial starvation, nor surgical stress with injury to the liver were thought to be important contributing factors. Evidence suggested that the reductions were related to alterations in carbohydrate metabolism which accompany rapid tissue growth and that coenzyme A may not play an important role in protein synthesis. 2. An unexplained rise in renal coenzyme A concentration was observed 24 hours after partial hepatectomy.

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Enzymic Action of Influenza Virus on Human Erythrocyte Stroma Components.* (23401)

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Sialic acid, a compound originally isolated from bovine submaxillary gland by Blix(1) is now recognized as an important constituent of soluble inhibitors of influenza virus hemagglutination. Its structure, proposed by Gottschalk(2), has only recently been established by the work of Heimer and Meyer(3). This chromogenic substance has also been identified among the dialyzable split products resulting from the action of influenza virus on certain mucoids(4). Interest in sialic acid in relation to action of influenza virus has led to a reexamination of receptor substances in human erythrocytes. The present study is based on earlier work(5) in which it was shown that virus receptors and blood group antigens could be concentrated from lyophilized erythrocyte stroma by relatively simple ultracentrifugation methods into a lipid-rich carbohydrate-containing fraction. This fraction has now been found also to contain a measurable proportion of chromogenic material giving absorption spectra in the direct

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† Markle Scholar in Medical Science.

Ehrlich and Bial reactions identical with those of crystalline sialic acid and certain soluble inhibitory mucoids. The erythrocyte chromogen has been recognized among the dialyzable split products resulting from the action of infective influenza virus on erythrocyte stroma and purified receptor substance derived therefrom(6).

Materials and methods. Influenza viruses were propagated by chorioallantoic inoculation of embryonated eggs 11 days of age, the fluid being harvested after 48 hours of incubation at 37°C.‡ Indicator strains were made by heating infective chorioallantoic fluid to 56°C for 2 hours. Hemagglutination inhibition tests were performed at room temperature, 5 hemagglutinating units for 0.1 ml of 2% chicken erythrocytes being the standard amount of virus in a final volume of 0.7 ml. End points were read by the pattern technic after approximately one hour. 0.9% sodium chloride, buffered to pH 7.2 with one part in 10 of M/15 phosphate buffer, was used as diluent. Nitrogen was measured by the Markham modification of the microKjeldahl procedure(7). Hexosamine was determined on hydrolyzed samples by the method of Elson and Morgan(8) and reducing sugar by that of Hagedorn and Jensen(9). Total phosphorus was determined by the method of King (10). Sialic acid was estimated by the direct Ehrlich and the Bial (orcinol) color reactions as described by Werner and Odin(11). Absorption at 560-565 in the direct Ehrlich reaction and that at 570 in the Bial reaction were used as the basis for calculating sialic acid content. Since the Ehrlich chromogen tended to fade rapidly, all readings were taken within 15 minutes of cooling. Crystalline sialic acid from bovine submaxillary mucoid(3)§ was used as a reference standard. Two soluble inhibitors of viral hemagglutination were also used as reference material. The first, human brain mucolipid (BML), was generously supplied by Dr. A. Rosenberg, and had been prepared by him according to methods already described(12). Human brain mucolipid contained 14-16% sialic acid and a minimum of 12-50 μg inhibited indicator PR8 and PR301. The second, sputum inhibitor (SpI), was prepared by Dr. B. F. Erlanger by the procedure of Marmion, *et al.*(13). It contained 4% sialic acid and a minimum of 2-10 μg inhibited several indicator strains. Total solids in dialyzed concentrated stroma were determined by drying measured samples to constant weight *in vacuo* over phosphorus pentoxide. Calcium borate buffered saline at pH 7.1-7.3 was prepared according to the formula of Burnet and Stone(14).

Preparation of stroma fraction containing virus receptor substance. Fresh human erythrocytes were washed 3 times in 0.9% sodium chloride solution, and hemolyzed with 9 volumes of cold distilled water. The precipitate obtained after adjustment of the hemolyzate to pH 5.5 with acetate was collected by centrifugation and brought to pH 7.4 and lyophilized. The lyophilized material was then redissolved in water at pH 7.4-8.0, brought to sodium chloride concentration of 0.15 M, and centrifuged in a model L Spinco ultracentrifuge, using either a 40 head (80,000 G) or a 21 head (40,000 G). The sediment was resuspended in 0.15 M saline, and recentrifuged 3 more times under the same conditions, the supernatant fluids being discarded. The last sediment (P4) was resuspended in a small volume of water and dialyzed against water before analysis. Certain preparations were then also dialyzed against calcium borate buffered saline for use in virus enzyme experiments. Analytical values for 4 preparations of P4 are given in Table I. The nitrogen concentration, taken as protein nitrogen, accounts for up to half the total weight. Values for reducing sugar and hexosamine are probably minimal, since conditions of hydrolysis other than those stated were not investigated. Phosphorus was measured in 3 preparations, and is present probably as phospholipid, which therefore would account for 27-30% of the total weight, using the usual factor of 25. The spectra of both the direct Ehrlich and the Bial chromogens corresponded precisely with those for sialic acid as shown in Fig. 1 and 2. All preparations gave a straw-colored opalescent solution in water,

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‡ The authors are indebted to Dr. Alice W. Knox for preparation of influenza virus.

§ Kindly supplied by Dr. R. Heimer.

TABLE I. Erythrocyte Stroma Fraction (P4) Containing Virus Receptor Substance.

Preparation No.	Preparation Donor	% nitrogen	% reducing sugar (as glucose)†	% hexosamine†	% chromogen Direct Ehrlich	% chromogen Bial	% phosphorus
1	Sch	8.0	4.5	1.8	2	1	1.2
2	Laz	7.5	4.5	2.1	2	2	1.1
3	Fri	6.8	4.8	1.8	2	2	1.2
4*	A	4.7	2.4	.9	n.d.	1	n.d.

* Preparation 6 in Table II.
† After hydrolysis (2N HCl, 2 hr) and neutralization.

and inhibited indicator virus hemagglutination in amounts of 25-100 μg.

Liberation of chromogen from stroma concentrate (P4) by concentrated influenza virus. Freshly harvested infective chorioallantoic fluid was centrifuged at 25,000 rpm (41,000 G) for 60-90 minutes, a 10- to 15-fold concentration of hemagglutinin being achieved by this procedure. The virus pellet was taken up in calcium borate buffered saline. Samples of P4 in amounts of approximately 0.5 g, following dialysis against calcium borate buffered saline or water, were then mixed with 70-140 μg total viral nitrogen. Appropriate controls were included, namely stroma with heat-inactivated virus (70° 10 minutes) or without virus, and active virus alone. Following incubation at 37°C for 16-18 hours, all samples were dialyzed against water and the dialyzates analyzed for chromogen by either direct Ehrlich or Bial reactions or both. The nondialyzable residues were heated to 70°C for 10 minutes to inactivate residual virus, and tested for hemagglutination inhibition with indicator strains. In the upper part of Table II, the results are given of a number of experiments in which 6 different preparations of P4 were treated with 5 different strains of active influenza virus. Loss of inhibitory activity, as

TABLE II. Effect of Concentrated Active Influenza Virus on Various Inhibitors of Hemagglutination.

Preparation	Strain of concentrated active virus	Chromogen liberated (as % of total) Direct Ehrlich	Chromogen liberated (as % of total) Bial	Loss of inhibitory activity for indicator PR8	PR301	FM1	Lee	GL
Erythrocyte stroma (P4)								
1	PR8	31	31	+	+	+	+	+
	PR301‡	33		+	+	—		
2	PR301‡	40		+	+	—		
3	PR301		31	+	+	—	+	+
No. 3 after treatment with PR301	FM1		26			+		
3	FM1		46	+	+	+	+	+
4	FM1		27	+	+	+	+	+
5	Lee	18	22	+	+	+	+	+
6*	GL		37	+	+	+	+	+
Erythrocyte stroma inhibitor (ESI)†								
1	PR301§		27	+	+	—	+	+
2	FM1		56	+	+	+	+	+
Sputum inhibitor (SpI)	PR8	26; 35‖		+		+	+	+
	PR301‡	31	36	+		+	+	+
Human brain mucolipid (BMI$_1$)	PR8	26; 27¶		+	+			
	PR301§		15	+	+			

* Preparation 4 in Table I. † Correspond with preparations 1 and 2, Table III. ‡ Same exp. § Same exp. ‖ 2 separate exp. ¶ Two separate exp.

indicated by a +, regularly represented a 50-fold or greater difference in inhibition titer between the material treated with active virus and the corresponding control, and frequently complete absence of inhibitor in the virus-treated sample. The controls regularly retained a full titer of inhibitor. In all instances, inactivation of the substrate was accompanied by the liberation of appreciable chromogen in dialyzable form, these amounts being expressed as a fraction of the total chromogen originally present in the reaction mixture. Control dialyzates consistently failed to give any color reaction. For purposes of comparison, sputum inhibitor (SpI) and brain mucolipid (BML) were included in experiments with PR301 and PR8. The results indicate that with these soluble materials as well destruction of or marked reduction in inhibitory activity as a result of viral action was accompanied by release of appreciable dialyzable chromogen. It is of interest that in 3 separate experiments with 3 different preparations of stroma P4, PR301 regularly failed to inactivate receptors for FM1; and in one experiment (preparation 3) subsequent treatment with FM1 of the substrate already treated with PR301 resulted in inactivation of FM1 receptors as well as in liberation of still more chromogen, *i.e.* 26% of what was left after PR301 treatment. Fig. 3 gives the absorption spectra of the dialyzable chromogen obtained with PR301 from erythrocyte stroma, sputum inhibitor, and brain mucolipid, with both the Bial and the direct Ehrlich reactions. With the exception of the dialyzable chromogen from preparation ES (Sch) P4, which in the Bial reaction gave a maximum slightly lower than 570, the curves correspond closely with those for sialic acid and the parent stroma fraction P4 as shown in Figs. 1 and 2.

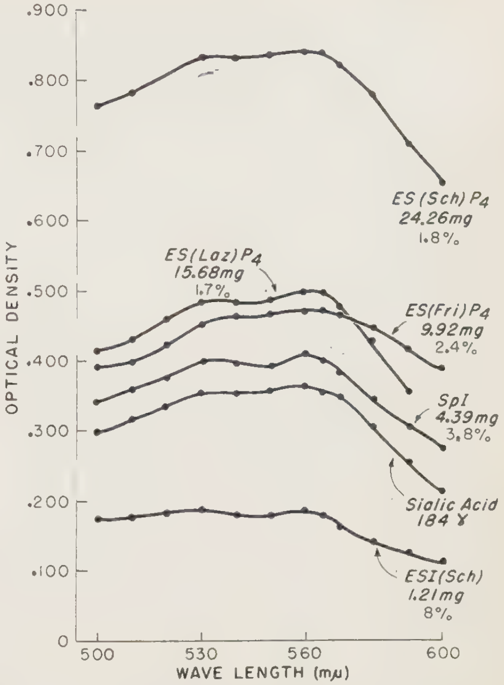

FIG. 1. Absorption spectra of chromogen (direct Ehrlich) in erythrocyte stroma concentrate (ES P4), erythrocyte stroma inhibitor (ESI) and sputum inhibitor (SpI), compared with spectrum of crystalline sialic acid.

Isolation of soluble erythrocyte stroma inhibitor (ESI). Since the relatively crude erythrocyte stroma concentrate (P4) described above contained appreciable lipid, the partition dialysis technic of Folch(15) was applied in a manner similar to that used by Rosenberg, *et al.*(12) for the isolation of brain mucolipid. In a typical preparation, approximately 2 g of P4 in the form of a thick paste were homogenized at 0°C for 2-3 minutes in a Waring blendor with about 400 ml chloroform-methanol (2:1, v:v). The whole mixture was then filtered, and the fil-

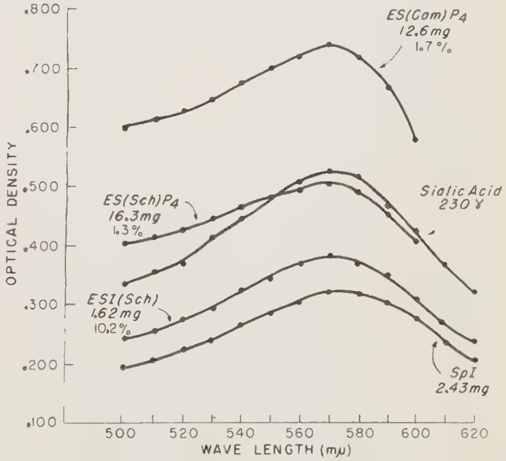

FIG. 2. Absorption spectra of chromogen (Bial) in erythrocyte stroma concentrate (ES P4), erythrocyte stroma inhibitor (ESI) and sputum inhibitor (SpI), compared with spectrum of crystalline sialic acid.

TABLE III. Erythrocyte Stroma Inhibitor (ESI).

Preparation*	% nitrogen	% reducing sugar (as glucose)†	% hexosamine†	% chromogen Direct Ehrlich	% chromogen Bial	% phosphorus	µg inhibiting 5 hemagglutinating units indicator PR301	FM1	Lee	GL
1	7.4	18	4	8	10	.5	5	4	9	9
2	8.5	19	6	7	11	n.d.	5	10	5	5

* Correspond with ESI Preparations 1 and 2, Table II.
† After hydrolysis (2N HCl, 2 hr) and neutralization.

trate dialyzed overnight against running tap water not exceeding 10°C. The aqueous layer was centrifuged clear and lyophilized. In 3 separate preparations the yield of white amorphous material, soluble in water, was 90-100 mg per liter of packed cells. Analytical data and inhibitory titers for several indicators with 2 preparations of ESI are given in Table III. Hexosamine and reducing sugar values are probably maximal, since samples hydrolyzed with 2N HCl for one to 5 hours gave essentially equivalent values. Electrophoresis of the first preparation at pH 8.6 in veronal 0.1 M showed the presence of one sharply defined major component, and 1-2 much smaller components.[‖] The major component showed viral hemagglutination inhibition activity comparable with that of the starting material. The absorption spectra obtained with this soluble erythrocyte stroma inhibitor (ESI) in the direct Ehrlich and Bial reactions are included in Figs. 1 and 2. In preparation 1, derived from a donor of blood group A_1, no blood group A antigen was detectable by inhibition of A-anti-A hemagglutination.

Liberation of chromogen from ESI by concentrated active virus. Two preparations of ESI were treated with concentrated infective influenza virus, one with strain PR301, the other with strain FM1, in the manner described in connection with the P 4 fractions. As shown in Table II, PR301 destroyed the inhibitory capacity for all indicators except FM1. This corresponds with the findings with P4 (Preparations 1, 2, and 3) in which the receptors for FM1 regularly survived treatment with PR301. Concentrated FM1, however, inactivated the inhibitor for all indicators tested. In addition, 27% and 56% of the chromogen was released in dialyzable form from these two preparations by the action of PR301 and FM1, respectively.

Chromatographic analysis. Limited chromatographic analysis of the dialyzable erythrocyte chromogen in several experiments was carried out, using two solvent systems and trichloroacetic acid-orcinol spray as the developing reagent(16). In the first system [iso-butyric acid-water-ammonia(17)] the Rf of ovine sialic acid[¶] was .24-.27, and that of the dialyzable chromogen split from both the P4 fraction and the soluble erythrocyte stroma inhibitor .21-.25. A second unidenti-

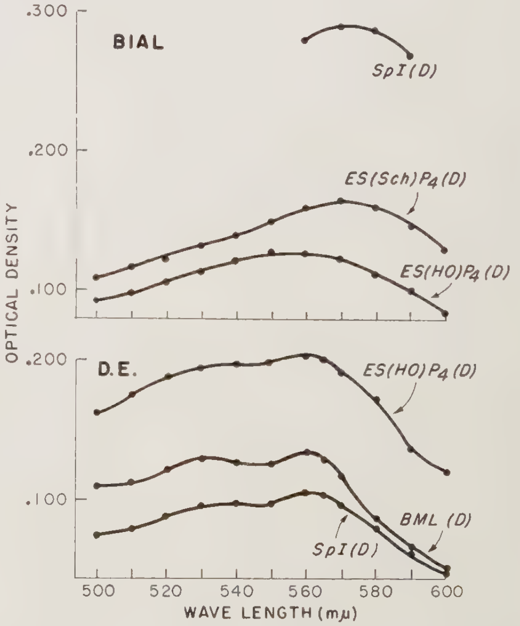

FIG. 3. Absorption spectra of dialyzable chromogen split from erythrocyte stroma concentrate (ES P4), sputum inhibitor (SpI), and human brain mucolipid (BML) by concentrated influenza virus, strain PR301.

[‖] Electrophoretic analysis performed by Dr. D. H. Moore.

[¶] Kindly supplied by Dr. A. Rosenberg.

fied component with an Rf value of .15-.16 was detected in 2 preparations. In the second solvent system [secondary butanol-acetic acid-acetone-water(18)], the Rf of ovine sialic acid was .44-.46, and that of the dialyzable chromogen .40-.41. The exact significance of these findings is not clear. They suggest, however, that the chromogenic substance split from stroma concentrate as well as from the purified ESI by viral action is some form of free sialic acid.

Discussion. The chromogen found to be liberated in dialyzable form from both crude and purified erythrocyte substrates by action of concentrated infective influenza virus is thought to be sialic acid. By analogy to similar enzymic action of influenza virus on certain soluble mucoids all of which are known to contain sialic acid, it is reasonable to infer that the same chromogen in stroma may be an integral part of the receptor site for the attachment of influenza virus to the intact cell surface. Klenk and Lempfrid(19) have recently reported isolation and crystallization of N-acetyl neuraminic acid [now among the substituted derivatives of neuraminic acid to be referred to as sialic acid(20)] from human erythrocyte stroma and its liberation from this substrate by purified receptor-destroying enzyme (RDE) of *Vibrio cholerae*. These authors have therefore concluded that the erythrocyte receptors for viral hemagglutination consist of sialic acid itself, assuming RDE to have enzymic specificities identical to those of influenza virus. The present studies, in accord with the findings of Klenk and Lempfrid, demonstrate clearly that enzymes of the infective influenza virus particle do indeed act on soluble human erythrocyte components in a similar manner, but the possibility that other complexes, including peptides with sialic acid linkage, might more reasonably serve as sites for attachment and action of *V. cholerae* and influenza virus enzymes has not been excluded. Such possibilities would still be consistent with the liberation of dialyzable sialic acid which, it should be noted, does not by itself inhibit viral hemagglutination. The question of the role of peptides in viral enzyme substrates is being investigated in this laboratory with the use of aminopeptidases of *Clostridium tertium*, culture filtrates of which also happen to show marked receptor-destroying activity(21).

Separation of soluble inhibitors of viral hemagglutination from human erythrocytes has been reported by other workers. DeBurgh, *et al.*(22), using lipid extraction procedures, isolated a fraction in small yield containing 2.6% nitrogen and 48% reducing substance. These authors gave evidence that, under certain conditions, the inhibitory substance was soluble both in water and in organic solvents. The present study also suggests that the purified receptor substance (ESI) includes or is actually a mucolipid, similar to the brain mucolipids described by Rosenberg, *et al.*(12). McCrea(23) isolated an inhibitor from human erythrocytes which gave hexosamine, reducing sugar, nitrogen and phosphorus values not dissimilar to those of the ESI described earlier. Both of the aforementioned investigations, however, were reported before the importance of sialic acid in relation to influenza virus enzymes was generally recognized.

It is of interest in the present studies that some suggestion of a receptor gradient was apparent, in that PR301 failed to inactivate receptors for FM1 either in crude stroma concentrate (P4) or in purified ESI, whereas FM1 inactivated receptors for both strains. Further studies will be necessary to test the validity of this interpretation, and to explore possible quantitative relationships between enzymic activity and receptor destruction in different strains of influenza virus. The greater sialidase(20) activity of RDE on erythrocyte stroma, as reported by Klenk and Lempfrid(19), compared with enzymes of influenza virus acting on similar substrates, as demonstrated in the present study, may be due to the lower total potency of the latter enzyme as used, or to the fact that RDE is soluble whereas influenza enzyme is in particulate form. It is tempting to speculate, however, that strains of virus may differ in the extent to which their enzymic components gain access to sensitive linkages in the receptor sites, whether because of spatial configuration of the substrate or peculiarities of the viral particle, or both.

Summary. The presence in human erythrocyte stroma of a chromogenic substance giving absorption spectra (direct Ehrlich and Bial reactions) identical with those of crystalline sialic acid has been confirmed. A potent water-soluble inhibitor of viral hemagglutination, containing 7-11% by weight of this chromogen, has been isolated by partition dialysis from concentrated human erythrocyte stroma. 30-40% of the chromogen in the crude stroma concentrate and up to 56% of that in the purified inhibitor was rendered dialyzable following treatment with concentrated infective influenza virus, which in most instances also destroyed receptors for indicator viruses. Limited chromatographic analysis indicated that the dialyzable chromogen is most probably sialic acid. These observations represent the first direct demonstration of split products resulting from enzymic action of influenza virus on human erythrocyte components.

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Growth of Human Tumors in Hibernating Hamsters.* (23402)

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(Introduced by R. O. Greep)
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Previous studies by Lyman and Fawcett (1) have shown that the growth rate of homologous tumor transplants in the hamster cheek pouch was diminished when the animals were induced to hibernate. The effect of the low temperature of hibernation (5°C) on the tissue of an animal which does not hibernate has not been previously tested. Lyman and Fawcett suggested that such a tissue might be less resistant to cold since there are apparently innate differences between the cellular physiology of hibernating and non-hibernating mammals. With the recent development of technics for transplanting human tumors

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to the cheek pouch of cortisone-treated hamsters(2,3) it has become possible to test the response of human tissues under the conditions of hibernation. During the past year we have tested 5 transplantable human tumors. Our results indicate that the growth of this tissue from a homeothermic mammal is markedly inhibited by hibernation, but that the tumor survives after prolonged exposure to these low temperatures. The possibility that the growth rate of these heterologous tumors might be slowed down sufficiently to provide a means of temporarily "storing" transplantable human tumors was our primary reason for carrying out this study.

Materials and methods. The tumors used in this study had been transplanted through many generations of hamsters over periods of 2 to 3 years(4), and with one exception were first transplanted in this laboratory from surgical specimens. The Pitt-41 strain was originally obtained from an autopsy specimen at the University of Pittsburgh.

Hamsters, 5 to 10 months of age, were kept in a cold room at 5° ± 2°C until they hibernated (see Lyman(5) for details). Tumor fragments one to 2 mg in size were then implanted in the submucosal space of each cheek pouch by means of trocar injection. In some animals a 50 mg pellet of cortisone[†] was implanted subcutaneously at this time, in others 2.5 mg of cortisone acetate suspension was injected by the same route. The latter group were given additional doses of 2.5 mg approximately twice a week according to requirements previously worked out for different tumor strains. The implantation technics invariably awakened the hibernating animals, but most of them soon returned to hibernation. The hamsters were checked daily thereafter and a light sprinkling of sawdust was dusted on the backs of the hibernating animals. If the sawdust were absent the next day, but the animal in hibernation, it was assumed that the animal had awakened and returned to hibernation. In calculating the total days in hibernation, such an animal was arbitrarily considered to have hibernated one-half a day. Tumors were inspected at intervals but no attempt was made to construct growth curves, since the usual pattern of growth at room temperature is quite predictable and our interest lay in the delay of growth during hibernation.

The hamsters were kept in the cold for 4 to 6 weeks and then moved to the warm room (23°C) where observations were continued until the tumors had either grown to the usual upper limits in size or obviously regressed. The animals were then sacrificed and the histology of all growing tumors confirmed by biopsy. A few transplants were made from these tumors to demonstrate that further propagation was feasible.

Results. The tumor strains tested will normally grow in at least three-fourths of the animals to nodules weighing 100 to 1000 mg (0.5 cm to 1.5 cm diameter). The growth period is 3 to 8 weeks; regression invariably occurs thereafter and metastases are not seen. The usual growth and the effect of hibernation upon it are reported below for each of the 5 strains.

(a) *Deac*—1 (Epidermoid carcinoma of the parotid gland). This tumor ordinarily grows to a diameter of 0.4 to 0.8 cm in 18 to 24 days and regresses completely in 30 to 42 days. In 2 test animals which hibernated more than 50% of the time, tumors grew only to 0.1 to 0.3 cm during a 4-week period in the cold. After 4 additional weeks at 23°C these animals had healthy tumors 0.42 to 0.73 cm in diameter.

(b) *Deac*—3 (Embryonal carcinoma of the testis). This ordinarily grows to a diameter of 0.8 to 1.2 cm in 21 to 28 days and begins to regress a few days later. Four test animals hibernated 49 to 74% of the 5 weeks, during which time no tumor grew beyond 0.25 cm in diameter although all were obviously vascularized. After the subsequent 3 weeks in the warm room, half of the tumors grew to average size.

(c) *Deac*—6 (Adenoacanthoma of the endometrium). This tumor reaches a diameter of 0.5 to 0.7 cm in 4 to 6 weeks with degeneration occurring soon thereafter. Typical normal growth to 0.45 cm in diameter was observed in one animal which hibernated only 2 days of the 5 weeks in the cold. Three other animals hibernated 64 to 76% of the 5 week period. The largest tumor at the end of this time was only 0.2 cm in diameter, but during the subsequent 3 weeks in the warm room all grew to the usual size.

[†] "Cortagen", furnished to us through the courtesy of the Schering Corp.

(d) *Deac*—8 (Adenocarcinoma of the endometrium). The usual period of growth for this tumor is 5 to 7 weeks with the tumor averaging 0.5 to 0.9 cm in diameter at the end of this period. The 4 test animals hibernated 43 to 72% of the 5 week period and no growth was apparent during this time, nor did growth occur during the ensuing 3 weeks in the warm room. The reason for this failure is not known. It is possible that this particular tumor tissue cannot tolerate such an exposure to cold, but more likely that it failed because of all our tumors it is one of the most slowly growing and difficult to maintain.

(e) *Pitt*—41 (Undifferentiated carcinoma of thyroid origin). The average period of growth is 14 to 21 days, with tumors reaching 0.9 to 1.5 cm in diameter. Five test animals hibernated 60 to 74% of the 5 weeks. Tumor growth was markedly delayed, with diameters averaging only 0.4 cm at the end of this period. Further transplants made from these tumors at this time grew successfully at the normal rate.

Discussion. Previous studies have shown that certain tissues from mammals which hibernate continue overt function at much lower temperatures than do similar tissues from non-hibernators(6,7). For example, an action potential can be elicited from the tibial nerve of the hamster *in vitro* at an average temperature of 3.4°C, while the same nerve in the rat ceases to function at about 9°C. This characteristic appears to be an innate difference of the animals in question, for there is no evidence that the ventral caudal nerve of the rat adapts to either high or low temperatures(8). Furthermore, it has been known for some time that mammals which hibernate can survive chilling to much lower temperatures than can non-hibernating mammals(9). Thus the hamster will recover from a deep body temperature of 3.8°C(10) while 18°C seems to be about the minimum temperature which the human can tolerate(11).

Although no quantitative comparison may be made with such a small sample, the heterologous tumors investigated in this study appeared to be less viable during hibernation than were the homologous tumors tested previously(1). However, most of the human tumors remained alive for many days in the cheek pouches of hibernating hamsters whose body temperatures are known to be less than 1°C above the environmental temperature of 5°C (12). Unless the host in some way changes the temperature tolerance of the implant, it would appear that human tumor tissue will survive well below the temperature where human tissue ceases all observable function.

As a means for "cold storage" of human tumor tissue, this technic is probably feasible. Rapidly growing strains which ordinarily require transplantation every 10 to 14 days could be transplanted only a third as often in animals maintained in the cold room. However, tumor storage in the frozen state is possible with many animal tumors and may prove more suitable for human material. Haemmerli and Toolan(13) have reported success in freezing a number of human transplantable strains for periods up to 6 months. We are investigating this problem at present and have had similar results.

Summary. Four human transplantable tumors have been grown in hibernating hamsters. One human tumor strain failed to grow. During hibernation tumor growth was markedly inhibited, but resumed its normal rate when animals were returned to warm room temperatures.

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Disappearance of Radioactive Iron from Plasma by Day and by Night. (23403)

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It has been shown that the diurnal variation of the plasma iron level is less evident in aplastic anemia, pernicious anemia in relapse, and in iron-deficiency anemia, and that full treatment of the 2 last conditions restores a normal diurnal variation. The diurnal variation was also found to be diminished and the plasma iron levels low in various conditions associated with increased erythropoiesis; polycythemia vera, congenital hemolytic anemia, nocturnal hemoglobinuria, pernicious anemia at the height of the reticulocyte response, iron-deficiency anemia treated with a limited quantity of iron(1) and in sickle-cell anemia (2). In similar conditions of increased erythropoietic activity, Huff *et al.*(3) found an increased plasma iron turnover. Under these circumstances the plasma iron level is low and remains relatively constant throughout the 24 hours. There is little or no rise in the level during sleep, possibly because the bone marrow is actively taking up iron during sleep. If this postulate be true it is reasonable to infer that the normal diurnal variation is brought about by diminished bone marrow activity during sleep and hence an increasing plasma iron level. In this respect it was especially interesting to find that the plasma iron level remained low throughout the 24 hours in the 2 patients with paroxysmal nocturnal hemoglobinuria, the hemolytic process in each case being compensated by increased erythropoietic activity.

Laurell(4) on the other hand noted the similarity between the diurnal plasma iron and bilirubin levels and explained both on the assumption that rate of destruction of hemoglobin is slightly greater during the night than during the day. It therefore seemed important to determine the fate of radioactive iron injected into the plasma by day and by night.

Method. The method adopted was similar to that of Wasserman *et al.*(5) except that the radioactive iron was converted to siderophilin by addition to the individual's plasma *in vitro*(6) and not by addition to Fraction IV-7 protein. Radioactive iron (^{59}Fe), in the form of ferric chloride, was neutralized with sterile NaOH and added to the plasma which was then allowed to stand for several hours before injection into the blood stream. After injection of radioactive iron, blood samples were collected by means of an in-dwelling needle in an antecubital vein and the subjects (except Subject 1) slept comfortably during the night experiments. Observations were made by day and by night (within a 24-hour period) on 3 normal individuals, one patient suffering from iron deficiency anemia and one from sickle-cell anemia. Diurnal plasma iron levels were determined by methods described previously(7).

Values for plasma iron turnover and the fraction of radio-iron removed per hour were calculated as in the paper by Huff *et al.*(3).

Results. For the 3 normal individuals T½ by day was found to be 96, 101 and 88 minutes and T½ by night 128, 126 and 126 minutes, respectively. The time-courses for Subject No. 3 are shown graphically in Fig. 1. In the patient with iron-deficiency anemia the clearance rates were almost identical by day and by night. Only one curve could be fitted, and T½ by day and by night was found to be 56 minutes (Fig. 2). In the patient with sickle-cell anemia the clearance rates were

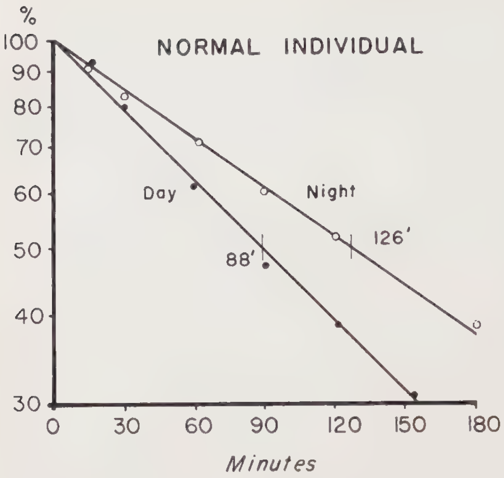

FIG. 1. Time-courses showing disappearance of radioactive iron from plasma by day and by night in normal individual.

found to be very rapid and were reversed so that the clearance by night was more rapid than the clearance by day; T½ by night was 33 minutes and T½ by day was 43 minutes (Fig. 3). Despite this patient's tall stature the measured blood volume exceeded the calculated volume. This finding has been noted by Smith(8) in patients with sickle-cell anemia during remission.

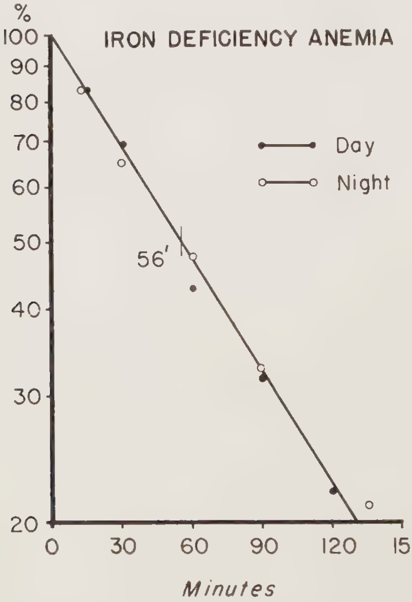

FIG. 2. Time-courses showing disappearance of radioactive iron from plasma by day and by night in iron-deficiency anemia.

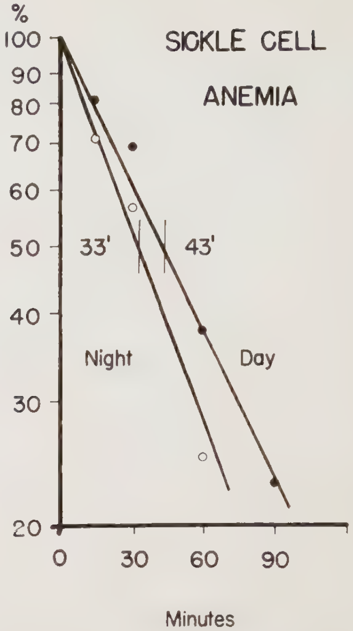

FIG. 3. Time-courses showing disappearance of radioactive iron from plasma by day and by night in sickle-cell anemia.

In neither of the last 2 cases was there any marked diurnal variation of the plasma iron level, whereas in the 3 normal subjects the diurnal variation followed the usual pattern of a rising level by night and a falling level by day (Fig. 4).

The calculated plasma iron turnover was

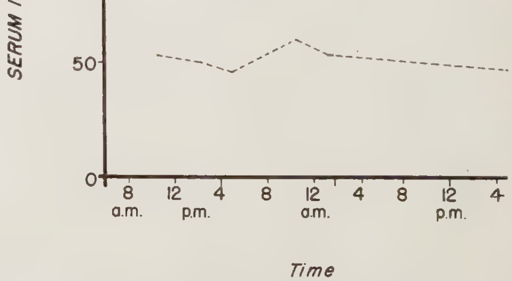

FIG. 4. Diurnal plasma iron levels. Solid lines—normal individuals (Subjects 1-3); dotted lines—upper, sickle-cell anemia (Subject 5), lower, iron-deficiency anemia (Subject 4).

TABLE I. Calculated Values following Injection of Radio-Iron by Day and by Night.

Subject and diagnosis	Sex and age	Time*	Plasma vol, ml	Plasma iron,† μg/100 ml	T½, min.	Iron fraction removed per hr	Plasma iron turnover, μg/hr
1. Normal	♂ 37	2 Day	3215	162 f	96	.432	2256
		1 Night		174	128	.325	1818
2. ”	♀ 42	2 Day	2280	170 f	101	.412	1596
		1 Night		96 r	126	.330	722
3. ”	♂ 19	1 Day	3240	142 f	88	.473	2174
		2 Night		129 r	126	.330	1380
4. Iron-deficiency anemia	♀ 23	2 Day	2630	50 f	56	.743	977
		1 Night		60 f	56	.743	1172
5. Sickle-cell anemia	♂ 18	1 Day	4690	105 r	43	.967	4762
		2 Night		105 s	33	1.26	6206

* Figures 1 and 2 refer to order in which experiments were carried out.

† Plasma iron levels at time of Fe^{59} admin. Determinations were also made 2-4 hr later, and the level was found to be falling (f), rising (r) or showing no change (s).

found to be decreased by night in the normal individuals with normal diurnal plasma iron variation, and increased by night in the anemic subjects who showed little or no diurnal variation. The findings are summarized in Table I.

Conclusion. These findings provide support for the hypothesis that the plasma iron level rises by night because of diminished utilization of iron for hemoglobin synthesis during sleep in the individual with normal erythropoietic activity. The conclusion is inferential in that no actual measurement of the newly formed hemoglobin was made but is clearly suggested by the findings of Wasserman and others(5) who interpret rate of removal of iron from the plasma as providing an index of erythropoietic activity. Bothwell and Mallett(9) found that plasma iron turnover varied throughout the day and was significantly affected by the plasma iron level. It is probable that an important consideration in assessing the findings of these authors concerns the state of the plasma iron level, *i.e.*, whether it is rising or falling at the time of plasma iron turnover determination. An interesting finding by Bothwell and Mallett was that the fraction of iron removed per hour from the plasma was uniformly large in both morning and evening determinations in anemic subjects.

The plasma iron reaches its lowest level just after sleep begins and rises during sleep (10). This diurnal pattern is reversed in night-workers who sleep by day(10-13). The usual rhythm, with morning levels higher than evening levels, is found only if the subject has slept during the night(13). The diminished plasma iron turnover and increased T½ by night suggest that diurnal variation of the plasma iron level is secondary to a diurnal variation in the rate of hemoglobin synthesis, which in turn is dependent upon the sleep rhythm. The high morning level of bilirubin in the plasma may be considered due to diurnal fluctuation in liver function rather than to increased hemolysis by night, as was held by Laurell(4). Josephson and Larsson(14) and Forsgren(15) studied diurnal excretion of bile by means of bile fistulae and found the quantity excreted by night less than that excreted by day.

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Anticonvulsant Activity of Some Substituted Ethylene Glycols. (23404)

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The clinical utility of diols such as mephenesin and 2,2-diethyl-1,3-propanediol (DEP) (I) has been limited by the short duration of action of these otherwise interesting drugs. Riley and Berger(1), and Berger and Ludwig(2) showed that rapid oxidation of the hydroxyl groups is responsible for this brief effect. Therefore it seemed desirable to synthesize structural variants less vulnerable to oxidative detoxication.

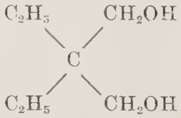
In this study a structural modification was considered favorable when the compound was effective against both electroshock and Metrazol convulsions at a dose below 100 mg/kg and had a duration of not less than 3 hours.

Methods. All compounds examined were administered orally as 5% gum acacia suspensions to Wistar-strain rats of either sex. The anticonvulsant activity of the compounds was measured at a predetermined time of peak activity by the maximal electroshock seizure test (M.E.S.) of Toman *et al.*(3), and by the subcutaneous Metrazol seizure test (Met.) described by Goodman *et al.*(4). The animals were observed before and after the convulsive challenges. Particular emphasis was placed on detection of motor impairment and other manifestations of central depression when present. Dose-response curves were established for active compounds whenever supply permitted. At least 3 dosage groups of 8 animals each were used to complete the median effective doses (ED_{50}) by the method of Bliss(5).

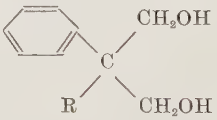
TABLE I. Effect of DEP Modification on Activity.

	$ED_{50} \pm$ S.E. (mg/kg)		
Compound	Maximal electroshock seizure	Metrazol seizure	Peak action (min.)
I (DEP)	154.4 ± 7.7	155.2 ± 8.4	30
II a	159.0 ± 9.9	229.3 ± 17.2	30–60
II b	142.8 ± 11.4	235.3 ± 30.6	30
III	120.7 ± 9.3	181.0 ± 15.4	30–90

Results. Table I shows the effect of modification of the DEP molecule upon anticonvulsant activity. Replacement of one ethyl group by phenyl (IIb) enhanced the depressant effect. A similar observation has been reported by Berger(6). In a further variation, the 1,3-diol arrangement of IIa was changed to 1,2-diol (III). Although this alteration diminished the depressant effect, the anticonvulsant activity of the new compound was superior to that of either I or II (Table I).

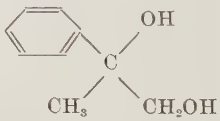


I (DEP)



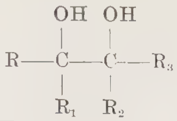
II

a) $R = CH_3$
b) $R = C_2H_5$



III

TABLE II. Effect of Glycol Substituents on Activity.



$$R-\underset{R_1}{\overset{OH}{C}}-\underset{R_2}{\overset{OH}{C}}-R_3$$

	R	R_1	R_2	R_3	ED_{50} ± S.E. (mg/kg)		Peak action (min.)
					Maximal electroshock seizure	Metrazol seizure	
1	C_6H_5	H	H	H	300	300	
2	”	CH_3	H	H	120.7 ± 9.3	181.0 ± 15.4	30– 90
3	”	C_2H_5	H	H	190.6 ± 21.5	173.8 ± 22.6	30
4	”	nC_3H_7	H	H	250	200	
5	”	C_6H_5	H	H	99.0 ± 11.1	200	30– 60
6	”	CH_3	CH_3	H	121.3 ± 20.0	137.9 ± 23.4	30–180
7	”	C_2H_5	”	H	119.1 ± 11.4	125.1 ± 12.5	30– 90
8	”	C_6H_5	”	H	107.6 ± 7.5	308.9 ± 38.6	30– 90
9	”	H	C_3H_7	C_3H_7	400	200	
10	”	CH_3	CH_3	CH_3	75.1 ± 10.1	150.5 ± 12.3	30–120
11	”	C_2H_5	”	”	100	69.2 ± 7.3	60
12	”	CH_3	C_2H_5	”	146.2 ± 8.5	88.7 ± 7.8	30– 90
13	”	”	C_3H_7	”	>160	73.5 ± 5.1	60
14	”	”	C_2H_5	C_2H_5	Tremors at 400 mg/kg		
15	Isoamyl	”	CH_3	CH_3	89.7 ± 9.4	86.3 ± 8.0	30
16	nC_3H_7	nC_3H_7	”	”	160	>160	
17	Cyclohexyl		”	”	100	>200	

In view of the enhanced anticonvulsant activity, systematic study of substituted ethylene glycols was undertaken.

In Table II may be seen the effect upon anticonvulsant potency afforded by alteration of the groups attached to the ethylene glycol nucleus. From these studies, 5 monophenyl substituted 1,2-diols were chosen and further altered by the substitution of halogen, methyl, or methoxy radicals in the phenyl ring. Completely substituted ethylene glycols are more potent anticonvulsant agents than tri-, di-, or monosubstituted analogues. The 1-phenyl-1-methyl configuration seems particularly desirable, for all 1-phenyl-1-ethyl compounds have short duration of action and the 1-1-diphenyl variants lack anti-Metrazol efficacy.

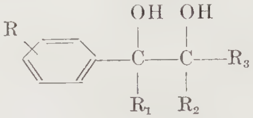
The optimal substitution of the second carbon atom of the ethylene glycols seems to be either monomethyl, dimethyl, or ethyl methyl. Larger groups cause loss of activity or other toxic effects, as observed in the diethyl compound No. 14.

Further modifications by substitution in the phenyl ring of compounds 2, 6, 10, 11 and 12 are summarized in Table III. In general, meta- or para-chlorination markedly improves anticonvulsant activity, whereas methyl or methoxy substitution reduces activity from that of the parent compound. Two analogues are of special interest. Compound 25, 2-*m*-chlorophenyl-3-methyl-2,3-butanediol, the most potent of the entire series, is equally effective as an anti-electroshock and anti-Metrazol agent. This equipotency is also observed in other meta-substituted phenyl compounds. Compound 26, 2-*p*-chlorophenyl-3-methyl-2,3-butanediol (phenaglycodol), though less potent than Compound 25, has the longest duration of action of the diols studied. It is devoid of manifestations of toxicity at effective dose levels. Studies by Slater *et al.* (7) indicate that this compound acts as a polysynaptic depressant.

Gruber and Mosier(8) have found the phenaglycodol to be an effective anticonvulsant. In addition, extensive clinical trials have shown that it is a useful neurosedative agent.

Discussion. The purpose of this investigation was the synthesis of structural variants of the DEP molecule that were longer in duration without sacrifice of potency. Phenaglycodol (2-*p*-chlorophenyl-3-methyl-2, 3-butanediol) is more potent and longer acting than DEP.

TABLE III. Effect of Aromatic Substituents on Activity.



	R	R_1	R_2	R_3	ED_{50} ± S.E. (mg/kg)		Peak action (min.)
					Maximal electroshock seizure	Metrazol seizure	
2	H	CH_3	H	H	120.7 ± 9.3	181.0 ± 15.4	30– 90
18	*o*-Cl	"	H	H	>125	>100	
19	*m*-Cl	"	H	H	111.9 ± 11.4	118.5 ± 14.2	30
20	*p*-Cl	"	H	H	>100	>100	
21	*o*-CH_3	"	H	H	>140	>400	
22	*m*-CH_3	"	H	H	144.2 ± 14.1	149.0 ± 23.8	30
23	*p*-CH_3	"	H	H	>250	>200	
6	H	"	CH_3	H	121.3 ± 20	137.9 ± 23.4	30–180
24	*p*-Cl	"	"	H	104.8 ± 11.5	73.8 ± 10	30–180
10	H	"	"	CH_3	75.1 ± 10.1	150.5 ± 12.3	30–120
25	*m*-Cl	"	"	"	35.8 ± 2.1	34.1 ± 4.3	60–180
26	*p*-Cl	"	"	"	63.0 ± 6.6	45.1 ± 7.7	60–240
27	*p*-F	"	"	"	77.9 ± 3.9	58.0 ± 6.7	30–120
28	*m*-CH_3	"	"	"	>100	>160	
29	*p*-CH_3	"	"	"	>100	>200	
30	*p*-OCH_3	"	"	"	>200	>250	
31	*m*-Cl	"	C_2H_5	"	92.4 ± 8.1	91.0 ± 10.9	30– 90
32	*p*-Cl	"	"	"	88.8 ± 7.4	74.8 ± 10.1	60–180
33	*p*-Cl	C_2H_5	CH_3	"	>250	>250	

This interesting activity seems dependent upon full substitution of the ethylene glycol nucleus. Such a configuration should reduce susceptibility to oxidative biotransformation. Current studies are directed toward elucidation of the metabolic fate of phenaglycodol.

Summary. 1. A series of substituted ethylene glycols has been studied for anticonvulsant activity by both the maximal electroshock seizure and subcutaneous Metrazol seizure tests. 2. Fully substituted ethylene glycols are more potent anticonvulsant agents than mono-, di-, or trisubstituted analogues. 2-*p*-Chlorophenyl - 3 - methyl-2, 3-butanediol (phenaglycodol) has been selected for clinical trial since it has high potency and long duration of action. It is suggested that the long duration of action may be related to full substitution of the ethylene glycol nucleus which should reduce the probability of oxidative detoxification.

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Specific Release of Heterogenetic "Mononucleosis Receptor" by Influenza Viruses, Receptor Destroying Enzyme and Plant Proteases. (23405)

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The usefulness of proteolytic enzymes in the detection of certain blood group antigen-antibody interactions(1) has led several investigators to study the applicability of trypsin and papain to a more reliable demonstration of mononucleosis antibody(2-5). Wöllner reported that papain destroys specifically the receptor for mononucleosis on sheep erythrocytes(4). On this basis, he developed a highly sensitive and specific diagnostic test for mononucleosis(6). Our results confirm the specificity of this test(5). They indicate, however, that enzymes besides papain are active and that at least part of the specific structure of the erythrocyte receptor for mononucleosis is not destroyed by enzymatic action, but released into the supernatant fluid(5).

This paper reports on specific removal of the erythrocyte receptor for mononucleosis by virus receptor destroying enzyme (R.D.E.) from *Vibrio cholerae,* by influenza viruses, and by the plant proteases bromelin, papain and ficin, and describes some serological and qualitative chemical properties of the material released by enzyme action.

Materials. Sera. Freshly drawn and clotted blood was obtained from patients with clinical, serological and hematological signs of mononucleosis. As controls bloods from healthy individuals and those suffering from a variety of diseases were included. Serum was removed by centrifugation, heat inactivated at 56°C for 30 min. and either tested immediately or stored at –15°C. *Erythrocytes*: A pool of citrated blood from 4 sheep (12 sheep used on a rotating basis) was obtained weekly and kept at 2 to 4°C. From this stock a 2% suspension was prepared each day after washing the erythrocytes 3 times with saline. Citrated beef erythrocytes were obtained from a slaughter house. *Solutions*: Saline solution (0.85%) and a m/15 Sørensen phosphate buffer of pH 7.2 were used. The final solutions were prepared as described by Pickles for the detection of incomplete antibodies (1).

Enzymes. The following enzyme preparations were employed: bromelin (Takamine); cathepsin (Delta); papain 1:200 (Fisher Scientific) both without and with glutathione (0.01 m final concentration); erepsin (General Biochemicals); ficin (Mann); ficin crystallized 3x (Worthington); pancreatin (Viscose Powder); rennet (General Biochemicals), chymotrypsinogen, crystallized 6x (Sigma Chemical); chymotrypsin (General Biochemicals); trypsin (Difco 1:250) carboxypeptidase crystallized 3x (Worthington); lipase (Delta Chemical); β-amylase (Bios); emulsin (Delta); hyaluronidase, 2 lots from bull testis (Wyeth); *Streptococcus hemolyticus* hyaluronidase (Wyeth); enzyme preparation from *Lactobacillus bifidus* variatio *pennsylvanicus;* lysozyme crystallized 3x (Worthington); Taka-diastase (Parke Davis); R.D.E. from *Vibrio cholerae* (Behringwerke, Germany) in concentration of about 300 units/ml (plus 0.015 m $CaCl_2$ final concentration). All enzymes were made up according to one of the two methods by Pickles(1) depending on the purity of the preparation.

Influenza viruses. Chick allantoic fluid containing the Melbourne strain of Type A, the Lee strain of Type B and the S_{15} strain of swine influenza virus, respectively (about $1x10^9$ I.D.$_{50}$ of virus per ml) was used in different experiments.

Glassware. All solutions were prepared in Pyrex glassware; 0.2 ml serological pipettes and test tubes of 7.5 x 0.9 cm size were used in all serological experiments.

Methods. Enzymatic treatment of erythrocytes: Sheep or beef erythrocytes, 1 to 7 days after bleeding, were washed 3 times, mixed with 5 volumes of the respective enzyme solution and incubated with repeated shaking for 30 min at 37°C. Subsequently, the cells were washed twice with saline. *Treat-*

TABLE I. Specific Removal of Receptor for Infectious Mononucleosis. Sera from cases with infectious mononucleosis (reciprocal titer).

		Serum unabsorbed		Serum absorbed with enzyme-treated sheep erythrocytes	
		Sheep erythrocytes			
Enzyme	Patient*	Native	Enzyme-treated	Native	Enzyme-treated
Receptor destroying enzyme	H.V.	64	16	64	<2
	A.M.	256	16	128	<2
	J.W.	>1024	16–32	512	<4
Influenza viruses:† (Melbourne strain of Type A or S_{15} strain of swine influenza)	W.J.	256	64	128	4
	J.W.	2048	256–512	512	8
	D.K.	128	64	64	<2
Bromelin	J.W.	2048	16	2048	2–4
	G.W.	512	8	128–256	<2
	H.D.	256	16	128	<2
Ficin (crude or 3 times crystallized)	J.H.	>128	16	128	<2
	G.W.	>256	8	64–128	<2
	J.W.	2048	128–256	512–1024	4
Papain (activation with SH groups is unnecessary)	G.W.	256	32	64–128	<2
	J.W.	2048	16	512	2
	C.D.	512–1024	64	2048	4

* Each enzyme was tested on serum of patient J.W. for comparative purposes.

† The Lee strain of Type B virus acted in a similar fashion, though weaker under the same experimental conditions.

ment of erythrocytes with influenza viruses. Packed and washed erythrocytes were incubated at 37°C for 12 to 20 hrs with a 30- to 40-fold volume of chick allantoic fluid containing influenza virus to which streptomycin and penicillin were added. The mixture was occasionally shaken and subsequently washed with saline. *Absorption of serum*: An equal amount of serum and packed sheep erythrocytes treated as described above were mixed well, kept at room temperature (22-25°C) for 1 hour and frequently shaken. After centrifugation, the erythrocytes were discarded and the serum was used for titration. Within each experimental series sheep erythrocytes treated with the same enzyme were used for absorption and titration.

Titration. Each serum was divided into 2 portions: unabsorbed and absorbed. For each of these, 2 geometrical dilution series (two fold) were set up. To each tube 0.2 ml saline was added and subsequently 0.2 ml serum to the first tube in each row. After thorough mixing, 0.2 ml was transferred from the first to the second tube and so forth. The same pipette was used throughout for each row. A volume of 0.2 ml of a 2% suspension of native erythrocytes was added to one of each of the 2 sets of dilutions of unabsorbed and absorbed serum and the same amount of enzyme treated erythrocytes was added to one of each of the other 2 sets of serum dilutions. The tubes were shaken, covered, and left overnight at room temperature. They were read macroscopically against diffuse light after a little shaking. The last tube showing unequivocal agglutination was taken as end point. Titers are expressed in terms of 2-fold geometrical dilution of serum. Final dilutions are obtained by multiplying these values by 2.

Controls. a) In each serological test were included 1) 0.2 ml saline plus 0.2 ml 2% suspension of native sheep erythrocytes; 2) Same as above except that the erythrocytes were treated with the enzyme under investigation; b) In the enzymatic studies, enzyme preparations boiled for 30 minutes and uninoculated allantoic fluid were included.

Hemagglutination inhibition. The inhibition method and controls employed have been described earlier(7), however 0.2 ml of all solutions or suspensions was used instead of 0.1 ml. After addition of inhibitor and also after addition of a 2% suspension of sheep erythrocytes, there was an incubation of about 10 hours at 4°C and room temperature re-

TABLE II. Increase of Agglutinin Titers for Receptors Other Than for Infectious Mononucleosis following Enzymatic Digestion.
Sera from cases without infectious mononucleosis (reciprocal titer).

		Serum unabsorbed	
		Sheep erythrocytes	
Enzyme	Patient	Native	Enzyme-treated
Receptor destroying enzyme	O.B.	64	256
	E.B.	4	8
	A.S.	4	8–16
Influenza virus	C.B.	2	8
	A.X.	2	4–8
Bromelin	O.B.	64	256
	E.B.	4	16
	A.S.	4	8–16
Ficin	P.P.	2	64
	E.B.	2	128
	A.S.	4	32
Papain	J.R.	2	16
	P.N.	128	1024
	P.D.	4–8	128–256

spectively. Readings were made with the naked eye. Blood group antisera and human blood group mucoids employed in this study have been described previously(7,8).

Preparation of enzymatic digests from erythrocytes. The supernate of enzymatically treated erythrocytes was boiled in a water bath for 30 min. Insoluble material was removed by centrifugation and the suitably concentrated solute tested as such or after dialysis through cellophane casing (Visking, average pore size 48 Å) for 72 hrs at 4°C against 60 volumes of distilled water (4 times changed). The color reactions employed in this investigation were standard laboratory procedures unless stated otherwise.

Results. Serological: Table I gives some typical serological observations. The enzyme preparations listed appear to remove the receptor for mononucleosis from the erythrocyte surface almost completely. This is evident, if one compares in unabsorbed sera from patients with mononucleosis the titer difference against native and against enzyme treated erythrocytes. Enzyme treated erythrocytes never gave a higher but usually a lower titer than native cells, due to removal of the receptor for the specific mononucleosis antibody. Sheep agglutinins which were not due to mononucleosis generally show an increase in titer against enzyme treated erythrocytes (Table II).

Unequivocal results were obtained uniformly if the sera were absorbed, prior to testing, with erythrocytes treated with one of the active enzyme preparations. Such a procedure removes all demonstrable agglutinins against sheep erythrocytes except those against the mononucleosis receptor. This is shown by the data on the right half of Table I.

None of the other enzyme preparations listed under "Materials" released the mononucleosis receptor or any sheep erythrocyte receptor reacting with agglutinins of human serum under the experimental conditions. All boiled enzyme preparations, boiled virus suspensions and uninoculated allantoic fluid were inactive.

The results of inhibition studies are listed in Table III. It can be seen that enzymatic digests from sheep and beef erythrocytes specifically inhibit the mononucleosis antibody. Beef cell digests inhibit to a larger extent than

TABLE III. Specific Inhibition of Infectious Mononucleosis Antibody.[*]

	Minimum amt of material giving complete inhibition of hemagglutination (mg/ml)
Sheep erythrocytes	
Papain digest	
non-dialyzable	1
dialyzable	5–10
R.D.E. digest	
non-dialyzable	5
dialyzable	inactive[†]
Influenza virus digest	
non-dialyzable	2– 5
dialyzable	5–10
Beef erythrocytes	
Papain digest	
non-dialyzable	0.3–1
dialyzable	± 10 mg
R.D.E. digest	
non-dialyzable	5
dialyzable	10
Controls	
Papain (boiled)	inactive
non-dialyzable	
dialyzable	
Influenza virus (boiled)	
non-dialyzable	
dialyzable	
R.D.E. (boiled)	

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[*] 4 minimum hemagglutinating doses.

[†] Inactive = No inhibition at 10 mg/ml or less.

TABLE IV. Color Reactions Given by Material Released Enzymatically from Erythrocytes.

	Reagents			
		Bial		
	Morgan-Elson without alkali	Neuraminic acid modification(9)	Molisch	Anthrone
Sheep erythrocytes				
Papain digest				
non-dialyzable	+++	++	++(+)	++
dialyzable	+	+	+	+
R.D.E. digest				
non-dialyzable	++	±	+++	+++
dialyzable	++	+	0	0
Influenza digest				
non-dialyzable	++	++	+++	+++
dialyzable	++	+	++	++
Beef erythrocytes				
Papain digest				
non-dialyzable	++	++	+++	++
dialyzable	+	+	+	+
R.D.E. digest				
non-dialyzable	+	+(+)	+++	+++
dialyzable	+	+++	+++	+++
Controls				
Mucoid from meconium	+++	+++	++	++
Papain solution				
non-dialyzable	—	—		
dialyzable	—	—		
Influenza virus				
non-dialyzable	±	—	+	+
dialyzable	+	—	+	+
R. D. E.	±	—	0	0
Allantoic fluid				
non-dialyzable	+	—	+	—
dialyzable	+	—	+	+

0 = Not determined because commercial R.D.E. contains 10% lactose as stabilizer.

those from sheep cells. In both instances, about 90% of the material subjected to digestion by papain and R.D.E. is dialyzable, whereas 90% or more of the inhibitor is not dialyzable. The inhibiting power of these crude preparations is low, but apparently specific. Human serum sickness and Forssman antibodies and blood group antibodies anti A, and B, anti H(O) and anti D(Rh_0) were not inhibited by these digests.

Human blood group mucoids A, B and H(O) from ovarian cysts and meconium and purified blood group substance from horse (starting material was Sharp and Dohme mucoid from horse, lot 346330) in concentrations of 10 mg per ml and below did not inhibit the mononucleosis antibody.

Chemical observations: The erythrocyte digests did not lose their activity by heating in a boiling water bath for 30 min. Besides boiling, dialysis and removal of insoluble matter, no fractionation or purification procedure was carried out. Reactions for carbohydrate which were performed on such solutions of comparable concentrations (about 1% dry weight) are shown in Table IV. They indicate the presence of neuraminic acid or its derivatives in these digests.

The biuret test on the non-dialyzable serologically active material was negative, while the ninhydrin reaction was weakly positive to positive. The active materials released by all enzymes investigated gave similar reactions.

Discussion. The fact that R.D.E. from *Vibrio cholerae* as well as influenza viruses and plant proteases remove specifically the erythrocyte receptor for mononucleosis poses the question as to mode of action of these enzymes and the nature of their substrate. The activity of influenza viruses and R.D.E. point to neuraminic acid or its derivatives as a structure possibly involved in the specific activity of the receptor, since the only enzyme influenza viruses are known to possess is neuraminidase(10). It is in agreement with this consideration that materials released during enzymatic inactivation give strong color reactions considered to be characteristic for neuraminic acid or its derivatives. Some of these color reactions are weakly positive when applied to normal allantoic fluid or allantoic fluid containing influenza virus. This may be explained either by material released from the allantoic membrane into the fluid or by substances contained in the influenza virus itself. The very weakly positive Morgan-Elson reaction (without alkali) in preparations of R.D.E. may have its origin in cholera vibrio, in the growth medium or in the enzyme stabilizer.

It is remarkable that proteinases from animals did not affect recognizably the mononucleosis receptor from erythrocytes while plant proteinases were active. It is unlikely that these proteinases contain an enzymatically active impurity because 3x crystallized ficin removes the receptor.

An immunological and presumably a chemical relationship may be postulated between the agent causing mononucleosis and the specific receptor on the erythrocyte surface, otherwise occurrence of a specific heterophilic antibody against sheep erythrocytes in sera of patients with mononucleosis would be difficult to explain. There is no indication that sheep erythrocytes are agglutinated by virus circulating in the blood stream of patients with mononucleosis. It is of interest that crude enzyme digests of sheep and beef erythrocytes inhibit the mononucleosis antibody. While the inhibition is not very high, it is nevertheless specific. Activity of these digests varied considerably in different experiments. Optimal conditions remain to be established.

The heat stability of the serologically active substances is in agreement with earlier observations by Schwarzweiss and Tomcsik (11), who obtained material capable of inhibiting mononucleosis antibody by extracting sheep and beef erythrocytes with hot organic solvents. The present experiments have shown in agreement with earlier authors(12) that beef erythrocytes yield more active inhibiting material on a weight basis, than those from sheep. Furthermore, our methods of preparation were entirely different from those of previous investigators.

The mononucleosis inhibitor is probably present predominantly in macromolecular form as it is essentially nondialyzable. Its heat stability and color reactions indicate that carbohydrate may be involved in the specificity of this receptor.

Summary. Release of heterogenetic infectious mononucleosis receptor from sheep and beef erythrocytes by receptor destroying enzyme, influenza viruses and plant proteases has been described. Numerous other enzymes were inactive. Material released by enzymatic action inhibited specifically infectious mononucleosis antibody and gave color reactions indicating that neuraminic acid or its derivatives are involved.

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Group A Hemolytic Streptococci. I. A Chemically Defined Medium for Growth from Small Inocula. (23406)

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Several attempts have been made to cultivate group A streptococci in chemically defined media(1-8). Slade, Knox and Slamp, and Slade and Slamp(7,8) obtained luxuriant growth of various strains of hemolytic streptococci by using media of 15 or 16 amino acids to which either a peptide preparation (CR14A) or heated crystalline ovalbumin was added; when, however, ovalbumin or the peptide preparation were omitted, no growth was obtained under defined conditions.

A defined medium allowing good growth and streptolysin 'O' production by various strains of group A streptococci has been described previously(9). The present study was performed to determine the nutritional factors necessary for optimal growth of group A streptococci. Details on streptolysin 'O' production in this medium will be reported elsewhere.

Materials and methods. Table I lists all components of the medium. Complete synthetic medium—COM-SYN-6—is composed of: basal medium, 22 amino acids and 6 vitamins. To study the nutritional demands of the various strains used, additional media were employed listed in Table I. All media were dispensed in 10 ml quantities into pyrex tubes 22 x 160 mm and were incubated at 37°C. Uninoculated tubes served as controls. Growth was determined as optical density (O.D.) and was measured with a 6A model Coleman Spectrophotometer at 550 mμ. Three strains of group A streptococci were used.† 1) Strain S84 (type 3) was obtained from the State Serum Institute, Copenhagen. 2) Richard's strain R 8191 (type 6) was obtained from the NCTC, London, and 3) Strain M, a local strain. *Inoculum.* Cultures maintained on blood agar were subcultured monthly. Single hemolytic colonies were inoculated into BHI medium and grown for 18 hours at 37°C. The bacterial growth was washed twice with an equal volume of phosphate buffer M/15, pH 7.2 made up in saline. Two consecutive passages in COM-SYN-6 medium were then performed using an inoculum of approximately 5 x 10^7/ml organisms (barely visible suspension, O.D. = 0.01). After 6-8 hours of incubation growth was diluted to give a barely visible suspension which was either further diluted or used as such; 0.05 ml aliquots were used to inoculate each assay tube. The medium was not considered adequate unless 2 consecutive passages in it without diminution of growth were obtained.

Results. Growth of different strains of streptococci in various media. Using a large inoculum (5 x 10^6 cells/ml) heavy growth was obtained in the following media: COM-SYN-6, CAS-AMINO-6, ENZ-AMINO-6, BHI, and Slade and Slamp medium(8). Cysteine HCl (100 μg/ml) was added prior to inoculation to all media except for BHI medium. Growth in BHI medium appeared earlier than in others and could be initiated from smaller inocula. No reduction in growth was observed even after 12 consecutive passages on all media used. *Influence of inoculum size on growth of Strain S84 on various media.* As seen in Table II, growth in BHI medium was initiated from as few as 500 cells. On the other hand, with COM-SYN-6 medium with added cysteine (100 μg/ml) or semi-defined media containing the same amount of cysteine, no growth from inocula smaller than 5 x 10^6/ml could be obtained. It was considered that the excellent growth obtained in BHI medium might have been due to its reducing and buffer properties as well as to its nutritional adequacy. Therefore, the effect of increasing concentrations of various reducing agents on growth from small inocula was tried. *Effect of increasing concentration of reducing agents on growth of streptococci.* Slade & Knox(6) stressed the importance of reducing agents for streptolysin 'O' formation on a semi-defined medium. Under their experimental conditions cysteine at a concentration of 75-100 μg/ml allowed optimal streptolysin 'O' production by the Richard's strain from an inoculum of approximately 1 x 10^7 organisms/tube (about 0.015 ml of O.D. of 0.25); concentrations of cysteine higher than 200 μg/ml were slightly inhibitory. However, we failed to obtain visible

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* This paper forms part of a Ph.D. thesis to be submitted.

† While this paper was submitted for publication 2 additional strains (c-203S and Blackmore) were grown successfully in the COM-SYN-6 medium. These strains were kindly supplied by Dr. A. W. Bernheimer, N. Y. University Medical College.

TABLE I. Composition of the Complete Synthetic Medium (COM-SYN-6).*

				"22 amino acids"			
Basal medium	mg/ml	"3 vitamins"	μg/ml	13 essential	μg/ml	9 non-essential	μg/ml
$Na_2HPO_4 \cdot 12H_2O$	3.4	Nicotinic acid	10	L-Arginine	75	L-Alanine	35
KH_2PO_4	.3	Pantothenate-Ca	"	L-Cysteine HCl†		L-Aspartic acid	120
$FeSO_4 \cdot 7H_2O$	.05	Riboflavin	"	Glycine	10	L-Asparagine	100
$MnCl_2 \cdot 4H_2O$	.025	"4 vitamins"		L-Glutamine	100	L-Cystine	70
$ZnSO_4 \cdot 7H_2O$	.01			L-Histidine	50	L-Glutamic acid	400
Glucose	4.0	Nicotinic acid	10	L-Leucine	50	DL-Isoleucine	200
$NaHCO_3$	.5	Pantothenate-Ca	"	L-Lysine	65	DL-Methionine	120
$MgSO_4$	.04	Pyridoxal · HCl	"	L-Phenylalanine	80	L-Proline	350
NaCl	.3	Riboflavin	"	DL-Serine	100	L-OH Proline	5
	μg/ml	"6 vitamins" (Essential and non-essential)		L-Tryptophane	200		
Guanine	10			L-Threonine	70		
Xanthine	10			L-Tyrosine	110		
Uracil	10	Biotin	.2	L-Valine	160		
		Nicotinic acid	10				
		Pantothenate-Ca	"				
		Pyridoxal · HCl	"				
		Riboflavin	"				
		Thiamine · HCl	.6				

* Other media employed:

1) COM-SYN-3 medium—basal medium, 22 amino acids, and 3 vitamins.
2) 13-SYN-6 " " " 13 " " and 6 " .
3) 13-SYN-4 " " " 13 " " and 4 " .
4) 13-SYN-3 " " " 13 " " and 3 " .
5) 12-SYN-6 " " " 12 " " (Cysteine omitted from 13 amino acids), and 6 vit.
6) 12-SYN-3 " " " 12 " " (as in 5), and 3 vit.
7) CAS-AMINO-6 " " " supplemented with 150 mg/10 ml casamino acid (Difco), and 6 vit.
8) ENZ-AMINO-6 " " " supplemented with 150 mg/10 ml enzymatic hydrolysate of casein (N.B.C.), and 6 vit.
9) BHI medium—Brain heart infusion medium (Difco).
10) Slade & Slamp medium—basal medium supplemented with 16 amino acids according to Slade & Slamp(8), and 6 vit.

† The amt of cysteine varied in different experiments; its concentration, therefore, is given separately. Cysteine was freshly prepared before inoculation. Glucose, vitamins, glutamine, bicarbonate, magnesium sulfate and cysteine were Seitz sterilized; all other materials were sterilized by steam pressure at 15 lb for 15 min. Solutions were made up with bidistilled water. The pH of the medium was adjusted to 7.6. All chemicals were purchased from NBC, Cleveland, Ohio.

TABLE II. Influence of Inoculum Size on Growth of Strain S84.

	Cells per ml				
Medium	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2
COM-SYN-6	.19	.07	.02	.02	.02
CAS-AMINO-6	.28	.11	.04	.02	.02
BHI	.32	.32	.32	.32	.28

Cysteine (100 μg/ml) was added prior to inoculation except for BHI medium. The inoculum consisted of cells grown for 6 hr in COM-SYN-6 medium; O.D. readings were performed after 18 hr of incubation at 37°C.

TABLE III. Effect of Increasing Concentrations of Cysteine HCl and Ascorbic Acid on Growth of Streptococci from Small Inocula.

Cysteine HCl, mg/ml	Cells per ml				
	2×10^5	1×10^5	1×10^4	1×10^3	5×10^2
.1	.02	.02	.02	.02	.02
.25	.18	.09	.02	.02	.02
.5	.26	.26	.24	.18	.15
.8	.32	.32	.32	.29	.23
1.2	.38	.35	.35	.29	.25
Ascorbic acid, mg/ml					
1.5	.12	.06	.02	.02	.02
2.0	.17	.12	.12	.02	.02
3.0	.22	.18	.15	.10	.02

The medium employed was COM-SYN-6. Cysteine and ascorbic acid were freshly prepared before inoculation; O.D. readings were taken after 18 hr of incubation at 37°C. The strain employed was S84.

growth from inocula smaller than 5 x 10^6 cells/ml in the presence of 100 μg/ml of cysteine. On the other hand, increasing the concentration of cysteine from 100 to 500 μg/ml allowed optimal growth from as few as 10^3 cells/ml. Even concentrations as high as 1200 μg/ml were effective in promoting growth from small inocula (5 x 10^2 cells/ml, Table III). Similar results were obtained with the 2 other strains employed. Ascorbic, thiomalic and thioglycollic acids were less effective than cysteine. *Specificity of reducing agents for growth of streptococci.* Koser & Thomas(10) using *Lactobacillus fermenti* found that the requirements for cysteine could be satisfied by ascorbic acid, while strains of *L. casei* and the majority of *L. plantarum* strains required cysteine for growth. Table IV shows that with the streptococcus employed ascorbic acid could replace cysteine only when a small amount (10 μg/ml) of cystine was present. Methionine, ethionine and thiosulfate were ineffective in the presence of ascorbic acid. Similar results were obtained with the 2 other strains used. *Nutritional requirements of streptococci in complete synthetic medium.* Omission from the complete medium (22 amino acids and 6 vitamins) of 2 vitamins (thiamine and biotin) and 9 non-essential amino acids, did not influence the growth of all strains employed. However, when both amino acids and vitamins were reduced to 13 and 3 respectively, the onset of growth was delayed to 36 hours (instead of 13-15 hr) and the final yield was about 25% of that obtained in COM-SYN-6 medium. Addition of pyridoxal to the 13-SYN-3 medium resulted in rapid and optimal growth after 15 hours (Table V). The need for pyridoxal in the 13 amino acids medium (13-SYN-3) in spite of its dispensability in the 22 amino acids medium (COM-SYN-3) suggested that non-essential amino acids may replace pyridoxal. The effect of different non-essential amino acids in a medium containing no pyridoxal was thus tried. It was

TABLE V. Growth of Strain S84 on Various Combinations of Amino Acids and Vitamins.

Medium	Hr of incubation			
	15	19	25	36
13-SYN-6	.23	.23	.23	.23
13-SYN-3	.05	.06	.06	.06
13- " + pyridoxal	.19	.23	.23	.23
13- " + DL-alanine	.19	.22	.22	.22
13- " + L-alanine	.18	.20	.23	.23

Concentration of cysteine was 500 μg/ml, of pyridoxal 10 μg/ml, of DL- and L-alanine: 70 and 35 μg/ml respectively. The inoculum consisted of 10^4/ml cells of a 5 hr culture grown on 13-SYN-4 medium. Growth was determined as O.D. at 550 mμ.

TABLE IV. Effect of Cysteine and Ascorbic Acid on Growth of Strain S84.

Medium	Cysteine•HCl, .3 mg/ml	Ascorbic acid, 3.0 mg/ml	Hr of incubation		
			15	19	24
COM-SYN-6*	+	—	.21	.23	.24
COM-SYN-3*	—	+	.22	.23	.23
12-SYN-6	—	+	.02	.02	.02
COM-SYN-6 + cystine, 10 μg/ml	—	+	.21	.23	.23
12-SYN-3 + " 10 "	—	+	.10	.10	.10
Idem + pyridoxal, 10 μg/ml	—	+	.18	.23	.24

* COM-SYN-6 and COM-SYN-3 contain cystine 5 μg/ml, but no cysteine. Growth was determined as O.D. at 550 mμ.

TABLE VI. Effect of Glutamine and Glutamic Acid on Growth of Strain S84.

Medium	Glutamine	Glutamic acid	Hr of incubation			
			13	15	19	36
13-SYN-6	—	—	.23	.23	.23	.23
” *	+	+	.22	.22	.22	.22
” *	—	+	.02	.05	.15	.23
” *	+	—	.20	.23	.24	.24

* 13-SYN-6 medium less glutamine. Concentration of glutamine and glutamic acid were 100 μg/ml. All tubes contained 400 μg/ml of cysteine. The inoculum consisted of 10^5/ml cells obtained from a 6 hr culture grown on 13-SYN-6 medium. Growth was determined as O.D. at 550 mμ.

found that both L- and DL-alanine effectively replaced pyridoxal when strain S84 and R8191 were used. With the M strain little or no growth was obtained in the presence of alanine.

Requirements for purines and pyrimidines. The complete medium contains guanine, xanthine and uracil. However, optimal growth was obtained when either one of the bases was used in a medium which consisted of 13 amino acids and 4 vitamins. Thus the findings of Slade & Knox(6) regarding interchangeability of purine and pyrimidine bases for growth of the Richard's strain was confirmed in a complete synthetic medium. Similar results were obtained with the other 2 strains employed. *Requirements for glutamine and glutamic acid.* Glutamine was necessary for growth of hemolytic streptococci and was non-replaceable by a series of compounds related to it(11,12). As can be seen from Table VI, optimal growth was obtained when the mixture of 12 amino acids and 6 vitamins was supplemented with glutamine and glutamic acid or with glutamine alone. In the presence of glutamic acid alone growth was considerably delayed and an optimum reached after 26 hours (in comparison to 13-15 hours in the presence of glutamine). Thus the importance of glutamine for growth of streptococci as reported by McIllwain *et al.* (11,12) is confirmed.

Discussion. Cultivation of group A streptococci in defined media is very much dependent on size of inoculum used, while no such limitations are encountered when media-like brain heart infusions are employed. With small inocula (less than 5×10^6/ml) no growth in defined media was obtained unless cysteine or other reducing agents were added. However, the amounts of cysteine needed were surprisingly high. An initial concentration of 600-1200 μg/ml produced growth from as few as 10^3/ml organisms, while 100 μg/ml cysteine allowed growth from not less than 5 $\times 10^6$/ml cells. Thus growth promoting conditions similar to those of BHI medium were obtained. The failure to cultivate streptococci in synthetic media from small inocula by other investigators was apparently due to an inadequacy of reducing conditions rather than lack of unidentified growth factors. It is conceivable that the heated crystalline ovalbumin used by Slade & Slamp(8) partly served to improve reducing conditions. It is difficult to explain the inability of cysteine to replace heated albumin unless this amino acid was used in insufficient amounts (concentrations of cysteine were not stated). However, the possibility is not ruled out that different strains possess different nutritional and reducing demands.

Cysteine was not only a superb reducing agent but also an essential amino acid for all the strains used. Ten μg/ml of either cysteine or cystine were adequate, provided a suitable reducing agent (ascorbic acid) was present. Another point of interest is the need for pyridoxal in a medium containing essential amino acids only (13-SYN-3). In fact, it was possible to replace pyridoxal by DL- or L-alanine. These results are in accord with the findings describing the replaceability of pyridoxal by DL-alanine(13,14,15). As L-alanine was also effective in replacing pyridoxal it seems plausible that the strains used (S84, R8191) possess an alanine racemase allowing utilization of the L-enanthiomorph (16).

Summary. 1) Defined media which allow

heavy growth of 3 strains of group A streptococci have been developed. The medium consists of either a) 21 amino acids, glutamine, 6 vitamins, salts, purines, pyrimidines and glucose or b) 13 amino acids and 4 vitamins. 2) Cysteine is important both as an essential amino acid and as a reducing agent. As an amino acid only a small amount (10 μg/ml) is needed and this can be substituted by an equivalent amount of cystine. As a reducing agent it can be replaced by ascorbic acid and less effectively by thiomalic or thioglycollic acids. Concentration of cysteine was critical for initiation of growth from small inocula. With less than 5 x 10^6/ml cells at least 350 μg/ml cysteine HCl are needed for obtaining visible growth. 3) Pyridoxal was necessary in a medium of 13 amino acids and 3 vitamins (nicotinic acid, pantothenate, riboflavin) whereas in a complete medium (22 amino acids) no need for pyridoxal was found. Pyridoxal could be replaced by L- or DL-alanine.

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Survival of Labeled Platelets in X-Irradiated Rabbits.* (23407)

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Although several excellent studies of radiation injury to animals have shown that the cause of the thrombocytopenia appears to be depression of platelet formation in the marrow(1,2,3), none of these studies showed the effect of radiation on a specific population of platelets. The development of a satisfactory method of labeling platelets made this type of study possible(4). This paper reports observations on the survival of Cr^{51} labeled platelets reinjected into rabbits irradiated with X-ray.

Methods and materials. 13 rabbits were exposed to 750 r of whole body X-ray, 24 hours after injection of autologous platelets labeled with sodium chromate^{51}. Twelve rabbits were subjected to the same procedure but with "sham" irradiation.

A Kellikoet X-ray machine was used set at 20 ma, 200 KV with HVL 1.1 mm of Cu using a 0.5 mm Cu filter. The unit was calibrated by the hospital physicist with a Victoreen 250 r chamber, and had an output of 12 r/min with back scatter. The tsd was 1 meter to the dorsal skin of the rabbit, and the animal was entirely within the 90% isodose line.

The platelets from 40 ml of rabbits heart blood were separated and labeled with 450-500 μc of sodium chromate^{51} (sp. activity 20-25 μc/μg) by the technic previously described(4). The labeled platelets

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* Supported by U. S. Atomic Energy Com.

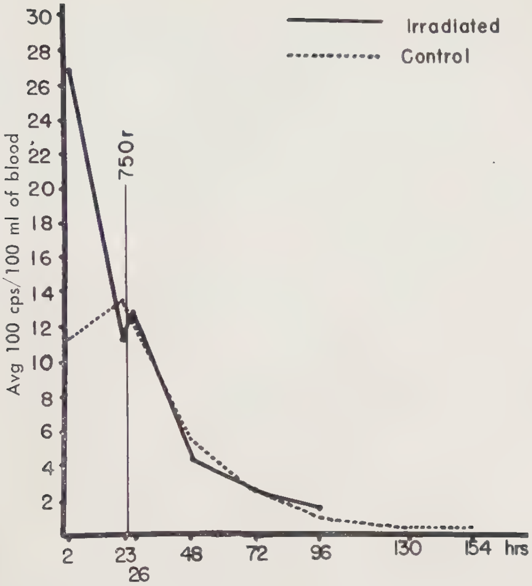

FIG. 1. Effect of 750 r X-ray on survival of Cr^{51} labeled platelets in rabbits. The higher initial value in the irradiated group is due to variations in the effectiveness of the initial tagging procedure (see ref. 4).

were washed once with 10 ml of the original plasma, reseparated, and suspended in the remaining plasma for reinjection into the rabbits' ear vein.

Ten ml blood samples were taken from the heart at 2, 23, 26 and 48 hrs after injection and daily thereafter as long as significant radioactivity persisted in the platelets.

Red blood cells were separated by slow centrifugation, and the platelets then separated by centrifugation at 2500 rpm for 30 min.

Since the amount of plasma radioactivity was negligible, the platelet button was measured directly after the removal of the platelet-poor plasma. All determinations were done in a well type scintillation counter and calculated as counts/second in 100 ml of blood.

Results. Survival of labeled platelets in rabbits exposed to 750 roentgens following reinjection of the labeled platelets was normal. The platelet survival curve of the irradiated animals showed a transient increase in amount of platelet radioactivity 2 hours after exposure (Fig. 1). The 2 curves are otherwise identical except for a higher initial level in the irradiated group due to variations in efficiency of labeling(4). The radioactivity present at 96 hours was sufficient to indicate a survival period of 6 days or more, as was actually demonstrated in a few of the control animals.†

Discussion. Studies on irradiation injury by others have shown the syndrome to have several phases. With very large doses of X-ray death occurs in a few hours. With somewhat smaller doses gastrointestinal hemorrhages and necrosis develop, 3-5 days after exposure. Hematologic effects become marked in the second week with thrombocytopenia developing after 5 days and reaching minimal levels between 8 and 10 days following exposure. Megakaryocytes disappear from the marrow about the 3rd-5th day. During the period of marrow depression the animal may die from hemorrhage or infection.

Lawrence rendered cats thrombocytopenic by irradiation and then restored their platelet counts by cross circulation with normal animals(7). The platelet count returned to the pre-transfusion levels in 2 to 4 days. Although no one has reported survivals of platelets in humans suffering from acute radiation thrombocytopenia, 4-6 day survivals of transfused unlabeled platelets have been reported in thrombocytopenia resulting from marrow failure due to idiopathic or toxic causes(8). In studying platelet life span in animals irradiated sufficiently to make them thrombocytopenic, the incidence of early death from intestinal necrosis, and the presence of hemorrhagic diathesis are hindering factors. If sufficient platelets are given to maintain hemostasis(9) the animals are no longer thrombocytopenic.

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† In our paper describing the method of labeling platelets with Cr^{51} we reported the measured survival of the tagged platelets in rabbits as 3-4 days (4). This short period was attributed to the volume of blood removed from the animals by frequent sampling. With improvements in technic, the use of sodium chromate^{51} of higher specific activity, and lowering the background radioactivity, the average survival of *in vitro* labeled platelets in rabbits is 6 days, a figure in agreement with the platelet life span reported by others using other methods of measurement(5,6).

In the pilot experiments for the present study, of 7 fasted rabbits given 600 r of X-ray and injected with labeled platelets after thrombocytopenia had developed, 2 showed a normal survival of labeled platelets, 3 animals died before platelet radioactivity had disappeared from the circulation, and 2 showed an early disappearance of platelet radioactivity coincidental with hemorrhage. When 3 larger rabbits, not fasted, were used, 2 doses of 750 r 2 weeks apart were required to produce persistent thrombocytopenia below 100,000/mm^3. None of these developed the intestinal necrosis syndrome, or generalized bleeding and injected labeled platelets survived normally for 5, 6, and 7 days. These studies, while incomplete, do not suggest any circulating anti-platelet factors resulting from radiation.

There is some evidence that irradiation in large doses has a damaging effect on circulating erythrocytes(2,10), in addition to the effect on erythropoiesis. The normal platelet survival reported here does not support such a contention with respect to platelets, at least with the doses of X-ray employed. No satisfactory explanation is available for the observed post-irradiation rise in circulating platelet radioactivity. It was present in 9 of 13 irradiated animals, and only 3 of 12 controls, but was not statistically significant. It was not accompanied by any change in the peripheral platelet count.

Conclusions. The life span of circulating platelets labeled *in vitro* with sodium chromate^{51} was normal in rabbits exposed to 750 r of X-ray subsequent to injection of the labeled platelets.

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A Modified Live Newcastle Disease Virus Vaccine.* (23408)

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Use of viable, virulent virus vaccines can increase incidence of respiratory disease in poultry, perpetuate disease in birds or produce a serious respiratory disease complex when superimposed in an already diseased flock. Failure to induce adequate immunity with inactivated Newcastle disease virus vaccine makes desirable an attenuated nonspreading virus strain; for vaccine production the strain should be propagable in nonchicken tissue to avoid contamination of poultry with egg-borne infections. This report describes modification of Newcastle disease (ND) virus and preliminary results of immunization of chickens.

Materials and methods. Modification of the virus. The California strain 11914 of Newcastle disease virus was modified by serial passage through tissue cultures composed of minced chicken embryo tissues suspended in Simm-Sanders medium containing bovine ultrafiltrate. Virus of passage 47 to 55 when

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administered by the air borne route to chickens in an enclosed chamber induced an excellent immunity, but on occasion caused symptoms or a mortality which reached 6.6% (1,2). Also parenterally administered virus killed 48-hour-old chicks. Investigations toward a solution of the respiratory disease complex reopened the search for a more desirable vaccine in 1954. Beginning with 47 passage, a series of 16 passages was continued using the same technic but alternating the passages through cultures containing bovine serum ultrafiltrate and cultures prepared without the ultrafiltrate. The series was continued for 12 passages using Sabin's technic in which the incubation period was reduced to 24 hours and the inoculum increased to at least 20% (3). A complete loss of virulence following parenteral inoculation into young chicks took place between the 58th and 75th passages. Intramuscular inoculation of 130 12-day-old chicks with the 71st passage virus was innocuous and larger amounts of the cultures were prepared for more extensive trials. Virus propagated in chicken embryo tissue was designated as line TC. To eliminate the use of chicken tissue, the 77th serial passage was propagated in quantities for study in monolayer tissue cultures of HeLa cells(4). The latter was designated as line TCH. *Titration of vaccine virus in embryos.* The inoculum employed (line TC or TCH) for vaccination consisted of the supernatant fluid of the culture which was frozen and stored in ampoules at –20°C. Aliquots of the infectious material were titrated in 8- or 9-day-old chicken embryonated eggs. Before use, the thawed suspension was diluted to the desired concentration with phosphate-buffered saline (pH 7.2).

Challenge virus. All chickens vaccinated with the TC or TCH modified virus were challenged intramuscularly at various intervals thereafter with strain GB Newcastle disease virus. The challenge for each bird consisted of 0.2 ml of infectious allantoic and amniotic fluids of chicken embryos diluted to contain 200,000 chicken embryo lethal doses (ELD_{100}). *Experimental chickens.* All chickens were obtained at one day of age and placed in a separate isolation building until used. Chicks from ND-susceptible dams were obtained from the University Poultry Husbandry flock which is not vaccinated and is under constant surveillance for disease. The commercially hatched chicks were obtained from breeding flocks with known vaccination histories. Hemagglutination inhibition (HI) and neutralization (SN) tests were conducted to determine the presence and degree of passive immunity in the chicks before each experiment(5).

TABLE I. Serological and Immune Response of 30-Day-Old Chickens Vaccinated Intramuscularly with 0.25 ml of Line TC Newcastle Disease Virus of the 76th Tissue Culture Passage.

	HI titers and challenge tests following vaccination				
	3 wk	7 wk		12 wk	
Vaccine titer ELD_{100}	HI*	HI*	Challenge†	HI*	Challenge†
10^6	7.0	3.3	0/3	1.6	0/3
10^4	8.0	2.8	0/3	1.2	0/3
10^2	4.0	1.7	0/3	1.0	2/3
None	1.0	1.0	3/3	1.0	3/3

* Expressed as geometric mean titer.

† No. susceptible/No. challenged.

Results. Immunogenicity of the virus. To determine optimum dosage of virus to induce an adequate immunity, 3 groups of 30-day-old chicks were inoculated intramuscularly with 10^6, 10^4 and 10^2 ELD_{100} doses respectively of the 76 passage of line TC. It will be observed in Table I that 10^4 and 10^6 ELD_{100} of virus produced a high degree of immunity which was effectively maintained for at least 12 weeks. Reducing quantity of virus resulted in a weaker immunological response and a shorter immunity. The results of 4 experiments demonstrating the avirulence of the strain TC and TCH for young chicks and the immunogenic properties of the virus are shown in Table II. In no instances were symptoms of the disease or any systemic reaction observed during the experiments. It is readily seen that one dose of .25 ml of line TC virus administered intramuscularly into 5-day-old chicks from susceptible dams resulted in a substantial HI antibody response, and all birds were solidly immune to an intramuscular challenge of virulent ND virus 12 weeks later. Progeny from immune dams in which HI and SN antibodies were demonstrated prior to vaccination acquired an irregular and

TABLE II. Immunological Response of Young Chicks of Susceptible or Immune Dams to Vaccination with Strains TC or TCH Newcastle Disease Virus.

	Vaccine				Weeks following vaccination†							
					3 wk		5 wk		8 wk		12 wk	
Exp. No.	Dosage* and age given	Virus and passage	Flock source of chicks	No. of birds	HI‡	Chal.‡	HI	Chal.	HI	Chal.	HI	Chal.
1	.25 ml IM at 5 days	TC 76th	Susceptible	23			8.6	0/14			4.7	0/ 9
2	*Idem*	″ ″	Commercial	38			1.03	10/11	1.07	3/10	1.08	7/17
3	.25 ml IM at 3 wk	″ ″	″	30			3.0	0/14	4.0	0/16		
4A	.2 ml IM at 6 wk	TCH 95th	Susceptible	20	2.5	0/10					1.10	0/10
4B	*Idem*	TC 76th	″	20	3.3	0/10					1.0	0/10

* Dosage: Adjusted to contain 10^4 embryo lethal doses/0.1 ml of suspension.
† At least 20% of animals in each experiment were held as controls. All were completely susceptible through the trials.
‡ See Table I (footnotes).

less effective immunity within the group. The influence of passive immunity on vaccination of young chicks with the modified virus was corroborated in Exp. 3. Chickens obtained from a commercial flock but held in isolation for 21 days before intramuscular vaccination gave a good serological response and showed a solid immunity to intramuscular challenge 8 weeks later. To determine the immune response produced in chickens by the modified ND virus propagated in HeLa cells (line TCH), the 95 passage of the latter was compared with the 76 passage of virus grown in chicken embryo tissues (line TC). Two groups, each containing 20 chicks, were vaccinated intramuscularly with 0.2 ml of each of the lines of virus. Exp. 4 of Table II illustrates that comparable serological responses were observed in the birds given the 2 lines of the modified virus and a solid immunity to intramuscular challenge was observed in all of the chicks 12 weeks after vaccination. In contrast, an equal number of nonvaccinated challenge controls suffered 100% mortality. *Duration of immunity.* Following the immunological response observed in young chicks, which lasted for a period that would be practical for broiler producers, the experiments were continued with the object of adapting the procedure for immunizing laying hens. The results are summarized in Tables III and IV. No respiratory or nervous symptoms of ND were observed in any of the birds receiving the virus. Birds vaccinated at 11, 20 or 5 weeks of age (Exp. 1B, 1C in Table III and 2 and 3 in Table IV) for the first time responded with a good antibody response and a solid immunity evident on challenge 4 and 8 weeks later. Administration of the second dose of 0.25 ml of the vaccine 9 weeks later (Exp. 1A, Table III) resulted in a marked anamnestic response in which the HI serum antibodies attained titers greater than those following a single injection into birds of varied ages and sources. The immunity following the second dose, expressed as a high antibody response and refractivity of the birds to intramuscular challenge, was still demonstrable 33 weeks after vaccination. This was the last time the birds were tested.

TABLE III. Duration of Immunity following Vaccination with Strain TC Newcastle Disease Virus.

			Weeks following first vaccination									
			4 wk		8 wk		24 wk		27 wk		33 wk	
Exp.	Dosage* and age given	No. of birds	HI†	Chal.†	HI	Chal.	HI	Chal.	HI	Chal.	HI	Chal.
1A	.25 ml IM at 11 wk .25 " " " 20 "	85	3.0	0/6	2.8	0/ 6	6.5 or >		3.7	0/10	4.0	0/10
B	.25 " " " 11 "	15					1.4		1.4	1/13		
C	.25 " " " 20 "	35	2.6	0/6	2.1	0/ 8	2.1	0/8				
D	None penmate controls	60	1.0	6/6	1.0	15/15			1.0	8/ 8	1.0	10/10

* See Table II (footnote *). † See Table I (footnotes).

The remaining chickens will be challenged at intervals to determine duration of immunity, and the complete study will be published elsewhere. There was no evidence of virus transmission from the vaccinated to susceptible-in-contact birds over a period of 33 weeks. Since no adverse effects were observed following exposure of approximately 900 chickens to the virus in the laboratory trials, the vaccine was administered to chickens on a commercial chicken ranch. In addition to the number of chickens reported in this paper, at least 20,000 chickens were vaccinated at 5 weeks of age without showing any symptoms of the disease. Of these, 12,000 were revaccinated when 12 to 14 weeks of age. Table IV gives the HI antibody titers of a representative number of birds following exposure to virus of 2 groups of 3750 chicks each. Revaccination with a reduced dosage of 0.2 ml (Table IV) did not result in as great a rise in HI antibody response; however, data from other experiments indicate that following vaccination a solid immunity to an intramuscular challenge dose of virulent virus can be demonstrated in the absence of serum HI antibodies. Nevertheless, more studies relative to dosage and the interim between inoculations are necessary to obtain the optimum immune response. It is also interesting to note that in each experiment the 150 birds left unvaccinated as pen contacts in each group (Exp. 2 and 3) did not show evidence of transmission of the virus from the vaccinated birds. Challenge data for these groups are not available at this time because of the limited number of controls and the desire to preserve for use at a later date. The avirulence of the modified virus (line

TABLE IV. Response of Chickens in Field to Vaccination with Modified Line TC Newcastle Disease Virus.

			HI response at weeks following first vaccination			
Exp.	Dosage* and age given	No. birds	4 wk HI†	8 wk HI	12 wk HI	Remarks
2	.2 ml IM at 5 wk .2 " " " 12 "	3750	3.7	1.5	2.8	First dose given when symptoms to infectious bronchitis vaccination were acute and severe.
	None pen contact controls	150	1.0	1.0	1.0	
3	.2 ml IM at 5 wk .2 " " " 14 "	3750	3.7	2.3	3.5 or >	
	None pen contact controls	150	1.0	1.0	1.0	
4	.2 ml IM at 12 wk	2500		3.0		Vac'inat'd during acute stages of chronic respiratory disease.
	None ranch controls	2500		1.0		

* See Table II (footnote *). † See Table I (footnote *).

TC) was also demonstrated when administered to birds experiencing an acute outbreak of another respiratory disease. In Exp. 4, 2500 12-week-old pullets were each injected with 0.20 ml of the vaccine and compared with an equal number of nonvaccinated birds under the same managemental conditions. Over a period of 12 days, no difference was observed in symptomatology, weight gains, or feed consumption between the two groups. Failure of symptoms to become aggravated was also observed in Exp. 2, in which the vaccination was conducted during an acute respiratory phase of infectious bronchitis induced by vaccination for the disease 10 days previously.

Summary. 1) Newcastle disease virus was modified by serial passage of the California 11914 strain through tissue cultures using minced chicken embryos suspended in Simm-Sanders medium. 2) The modified virus was administered intramuscularly to over 20,000 chickens of all ages without producing any nervous or respiratory symptoms, but produced an immunity as demonstrated serologically and by intramuscular challenge with a virulent strain of ND virus. One dose of the vaccine given to susceptible chicks at 5 days of age or over induced an immunity lasting at least 12 weeks. Two doses from 7 to 9 weeks apart have resulted in a high degree of immunity which was still evident 33 weeks later. 3) When administered to 2 flocks of chickens, one in acute stage of infectious bronchitis and the other with chronic respiratory disease, the virus apparently did not increase the severity of the respiratory disease condition. 4) Repeated trials showed that vaccinated chickens did not transmit the infection to unvaccinated susceptible birds within the same pen during 33 weeks. 5) The modified virus can be propagated on HeLa cells to produce a vaccine for poultry, thus avoiding the introduction of egg-borne infections to poultry flocks through vaccines produced in chicken embryonated eggs.

The author wishes to express his appreciation for the fine technical assistance of E. E. Stuart, R. Corstvet and J. Fabricant.

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Some Physical and Chemical Properties of Antibody Against Complement.* (23409)

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We(1) have previously described an antibody against complement. Most of the experiments then reported were concerned with activity of anticomplement as an inhibitor in the immune hemolytic system. Mention was made, however, of experiments to determine more of the physical and chemical properties of this antibody. The purpose of this paper is to describe in more detail studies on quantitative antibody protein, antibody expressed as carbon-14, Tiselius electrophoretic analyses, and fractionation of anticomplement-containing sera.

Methods. Antibody was prepared in rabbits to complement of the guinea pig, human being, monkey, and mouse by the method previously described(1). Briefly, the immunizing procedure consisted of giving animals several subcutaneous injections of different immune precipitates to which complement had

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TABLE I. Anticomplement Protein Determination.

Material analyzed	Avg total protein (mg)	Difference from ppt.	Diff. from ppt. + C′
Precipitate* alone	5.60		
" + C′†	5.90	+.30	
" + △C′‡	5.70	+.10	−.20
" + C′ + RAGPC′§	6.25	+.65	+.35
" + △C′ + RAGPC′	5.85	+.25	−.05

* Bovine serum albumin + anti-bovine albumin. † Complement. ‡ Complement heated at 56°C, 1 hr. § Rabbit anti-guinea pig complement.

been affixed, incorporated in an adjuvant.

Since antibody to complement was prepared by immunization of animals with immune precipitates to which complement was adsorbed, this antibody should combine specifically with such complexes and be measurable as increased protein in the aggregate. On the other hand, if precipitates were treated with heat inactivated complement, no specific complement protein, but only nonspecific protein combination, should be measurable, as shown by Heidelberger(2). Combination of anticomplement with the latter complexes should therefore reflect nonspecific protein binding from antibody-containing sera. The difference should give a quantitative measure of anticomplement uptake in a given set of experimental conditions.

For the expression of anticomplement as antibody protein, a modification of the method used by Heidelberger(2) for determination of complement as combining nitrogen was used. Precipitates were prepared from crystalline bovine serum albumin and rabbit anti-bovine albumin in the zone of slight antibody excess. Triplicate sets of tubes were used for each variable. After allowing the precipitates to form for 15 minutes at room temperature, neutralized fresh guinea pig complement, complement heated at 56°C for 60 minutes, or 0.15 M NaCl was added to each set of tubes. The mixtures were then incubated for 16 hours at 4°C. Anticomplement or saline was then added. Following incubation for 30 minutes at room temperature, all tubes were centrifuged 30 minutes at 4°C and 3,000 rpm. Precipitates were washed 3 times with cold 0.15 M NaCl under the same conditions of centrifugation. After the final washing, sediment in each tube was resuspended in saline, and quantitative biuret determinations made by the method of Gornall, Bordawill, and David(3).

Typical results of experiments with anti-guinea pig complement are described in Table I. The increase in protein due to combination of complement with the precipitate is 0.3 mg/3.0 ml guinea pig serum. Of this amount, 0.1 mg would be due to nonspecific adsorption, as evidenced by the combination of heated complement. Addition of 2 ml of anticomplement to the precipitate-complement complex accounted for the addition of 0.25 mg more protein. If heated complement were used, however, the amount of non-specific protein combination from anticomplement-containing sera amounted to 0.15 mg protein.

Correcting for non-specific adsorption and for volume of materials added, one finds that the total amount of complement protein bound to the precipitate is 0.067 mg/ml of guinea pig serum. The amount of antibody protein fixed to the precipitate-complement complex, on the other hand, is 0.1 mg/ml of antiserum.

Another means of showing the combination of anticomplement with its specific antigen would be measurement of uptake of radioactivity if the antibody could be appropriately labeled. Preliminary experiments(1) showed that carbon-14 labeled antibody which had combined with the complement-precipitate complex gave counts significantly above those obtained when C^{14} labeled normal rabbit serum was used for control. Non-specific adsorption of C^{14} from other serum fractions caused counts to be considerably above background in control tests, however.

In the present experiments, rabbits immunized against guinea pig complement were maintained for approximately 3 months to al-

TABLE II. Anticomplement Expressed as Carbon-14.

Property measured	Ppt + C′* + RAGPC′(C^{14})†	Ppt + C′ + NRS(C^{14})‡	Difference
Total carbon, mg	2.793 (2.780)§	2.584 (2.584)	.196
	2.784	2.618	
	2.752	2.550	
Counts/min. (corr.)	203.4 (204.9)	109.1 (118.7)	86.2 (72%)
	210.2	129.7	
	201.0	117.2	
Counts/min./mg C	72.8 (73.8)	42.2 (45.9)	27.9 (60%)
	75.5	49.5	
	73.0	45.9	

* Precipitate + complement. † Rabbit anti-guinea pig complement. ‡ Normal rabbit serum. § Values in parentheses are avg.

low a decline of antibody titer. Following this period each animal was given a "booster" injection of homologous complement antigen subcutaneously without adjuvant. Five days later, when the anamnestic response was in the ascending phase, each immunized animal and 2 normal control rabbits were given 9.18 mg of glycine-l-C^{14} by intravenous injection. The glycine contained a specific activity of 13.8 μc/mg. Six hours after receipt of the radioactive glycine all animals were exsanguinated by cardiac puncture and their serum separated for study. Both the serum containing labeled anti-guinea pig complement and the labeled normal rabbit serum were fractionated in a Misco Electrochromatography Apparatus to minimize non-specific adsorption. Material from tubes containing high anticomplement activity as measured by inhibition of immune hemolysis was pooled, as was material from the same tubes containing fractions of normal serum. Precipitates were prepared in the same manner as previously described for protein determination, and 1.0 ml of guinea pig complement was adsorbed to the precipitate in each tube. Aliquot portions of either the pooled fractions of anticomplement or normal rabbit serum were added. The precipitates were allowed to incubate overnight and then washed and centrifuged. Each precipitate was analyzed for total carbon and carbon-14 activity by the gas phase counting method of Van Slyke, Steele, and Plazin(4).

The results of studies on C^{14} uptake by precipitate-complement complexes from fractions of such sera are presented in Table II. The average increase in total carbon uptake from anticomplement-containing sera is 0.196 mg above the uptake from normal serum. The average increase in carbon counts per minute is 86.2, or a 72% increase.

Even after separation the amount of C^{14} uptake from normal rabbit serum (172.8 counts/min, uncorrected) was considerably above the average background (72.9 counts/min.). The count for precipitate-complement complexes to which anticomplement was affixed (250.5 counts/min, uncorrected), was sufficiently increased to rule out the possibility of non-specific adsorption as the cause of the elevation in C^{14} activity.

Additional evidence in support of the characterization of anticomplement as true antibody would be the changes in serum protein content following immunization with complement antigens. Serological studies to detect antibodies for soluble protein antigens used to prepare the precipitates and for guinea pig serum were negative. The only observed change in sera of such animals was a rise in complement-inhibiting ability.

Tiselius electrophoretic studies were performed on sera of individual rabbits immunized against guinea pig, mouse, and human complement, before and after the immunizing process. A Perkin-Elmer Electrophoretic Apparatus was used. Barbital buffer, pH 8.6, ionic strength 0.1, resistance 330 ohms, was employed for all determinations. The time of analysis was 70 minutes, the current 10 ma, and the temperature 1°C.

The percentage values for each protein fraction before and after immunization in individual animals are given in Table III. An increase between 8.7 and 10.8% in γ

TABLE III. Electrophoretic Percentages of Protein Fractions before and after Immunization.

	RAGPC′*			RAHC′†			RAMC′‡		
Fraction	Pre	Post	% change	Pre	Post	% change	Pre	Post	% change
Albumin	51.3	38.4	− 12.9	57.6	57.1	− .5	57.7	48.0	− 9.7
α_1 globulin	7.1	6.6	− .5	4.9	2.7	− 2.2	5.0	4.5	− .5
α_2 "	18.6	14.8	− 3.8	6.7	6.5	− .2	6.7	7.8	+ 1.1
β "	16.8	24.0	+ 7.2	19.9	14.1	− 5.8	21.8	20.1	− 1.7
γ "	6.2	16.2	+10	10.9	19.6	+8.7	8.8	19.6	+10.8

* Rabbit anti-guinea pig complement. † Rabbit anti-human complement. ‡ Rabbit anti-mouse complement.

globulin occurred in the serum of each animal following immunization. In the rabbit anti-guinea pig complement, an increase in β globulin was also noted which appears to be due to the concentration of hemoglobin in this area. Although we realize that numerous factors may affect the γ globulin concentration, the consistent increase of this fraction in these animals following immunization in the absence of other obvious stimuli would tend to support the nature of anticomplement as true antibody.

For certain analytical procedures (carbon-14-analysis), and for *in vivo* studies, it was necessary to obtain anticomplement in a purified and concentrated form. Fractionation of anticomplement-containing sera was carried out in a Karler-Misco Electrochromatography Apparatus using 0.01 M barbital buffer, pH 8.6, ionic strength 0.01, at room temperature. The rate of serum input was 0.5 ml per hour. The total fluid volume in each fraction tube was estimated. Total protein per fraction was determined by the quantitative biuret method(3). The antibody titer of each fraction was measured by its ability to prevent 2 full units of complement from hemolysing sensitized sheep erythrocytes.

The efficiency of protein recovery in the process was 54.4% of total input. Antibody was demonstrable to some extent in 13 of the 22 fractions recovered. These contained 64.8% of the recovered protein. However, most of the antibody activity was found in 9 fractions containing 45.6% of the protein. As expected, maximum activity occurred in the fractions where γ globulin would normally separate out.

Summary. 1) Our data indicate that antibody against complement can be expressed as protein in specific combination with an immune precipitate to which complement has been affixed. 2) Anti-complement which has been labeled by injection of glycine-l-C^{14} into animals during active antibody production can be demonstrated as carbon-14 bound to precipitate-complement complexes. 3) Electrophoretic analysis of the sera of rabbits before and after immunization with guinea pig, human, and mouse complement shows that a consistent rise in γ globulin occurs during the immunization process. 4) Finally, electrochromatographic separation of antibody containing sera proved to be an effective means of concentration and partial purification of the antibody.

We wish to express our gratitude to the staff of the Medical Department at Brookhaven National Laboratory for their kindness in instructing us, assisting, and allowing the use of their equipment for a portion of this study.

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C^{14} Thyroxine Transport in Thoracic Lymph in Rats.* (23410)

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The thyroid hormone must become part of the cell's environment to exert its peripheral metabolic action. The entrance of the hormone into the circulation and subsequent binding must therefore be followed by its escape into the extravascular extracellular fluid which bathes peripheral cells. Salter *et al.*(1) and Salter and Johnson(2) emphasized the importance of binding "hormonal iodine" to the smaller molecular sized protein fractions of plasma and these would probably escape through the capillaries into the lymph carrying with them relatively rich amounts of "hormonal iodine" for the peripheral cell environment.

The present study is concerned with the transport mechanism and character of the hormone in thoracic lymph using DL-thyroxine-l-C^{14}.†

Methods. Adult male rats of the Sprague-Dawley strain weighing about 225 g were used. All animals were maintained on Rockland Complete Rat Diet and fed *ad libitum.* Under ether anesthesia, the thoracic lymph duct was cannulated through a left lateral abdominal incision using a polyethylene tube (inside diameter, 1.10 mm) according to a modification(3) of Bollman's technic(4). The abdominal opening was closed and sealed with collodion and the animal placed in a restraining cage. When the lymph reached the end of the tube (volume of 0.2 ml), the animal was injected subcutaneously in the right flank with 780 μg (one μM) of DL-thyroxine-l-C^{14}/kg body weight. From 8 animals, 8 hourly lymph samples were collected in 2 ml pipettes which had been previously cut off at the lowest graduation and closed with a rubber-dam. One drop of heparin was added to each collecting pipette to prevent coagulation of the sample. Lymph radioactivity was determined by drying 0.1 ml aliquots on copper plates and counting them under a thin mica window Geiger counter. *Plasma radioactivity levels* were determined in 8 animals from blood collected by 8 hourly heart punctures. About 0.4 ml of blood was obtained at each collection period and duplicate 0.05 ml samples of plasma were plated and counted in the same manner as for lymph. *Chromatographic analysis* of lymph was made using modification of the method described by Rosenberg(5). Cellulose columns 85 cm in height were prepared using Solka-Floc BW 200. The running solvent contained n-butyl alcohol, glacial acetic acid, and water in proportions of 75:10:15, respectively. Regulation of the solvent flow rate to 0.5 ml/hour was attained by connecting the 300 ml solvent container to top of column. Pooled butyl alcohol extracts of the most active samples (2-4 hours) of lymph were used. Approximately 1 to 2 μM each of thyroxine, triiodothyronine and diiodotyrosine was dissolved in 0.1 ml of running solvent and added to top of column as a carrier and for position identification. Effluent samples of 0.5 ml were obtained using an automatic fraction collector and radioactivity measurements were made in the same manner as for lymph and plasma. The presence and relative concentrations of each of the known constituents were determined by the photometric ninhydrin method of Moore and Stein(6).

Results. From Fig. 1 it is seen that over 3% of the injected dose of C^{14} labelled thyroxine appeared in the thoracic lymph after 8 hours. There is nearly a straight line relationship between the accumulation of activity and time.

Fig. 2 compares the radioactivity present in plasma and lymph. Radioactive thyroxine appeared in both fluids within the first hour, peaking in about 1-3 hours, and then grad-

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* This investigation was supported by grant from N.I.H., U.S. Public Health Service.

† The C^{14} used was obtained from Oak Ridge National Laboratory on allocation from Isotopes Division, U. S. Atomic Energy.

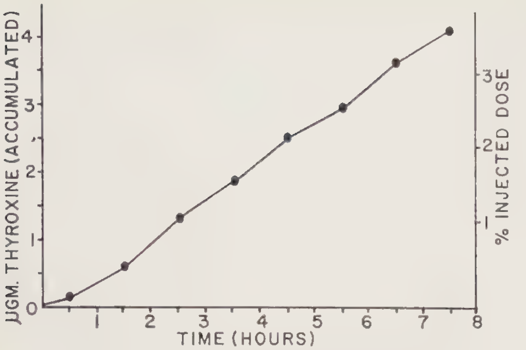

FIG. 1. Accumulated lymph radioactivity after a single subcut. inj. of 0.78 mg/kg of thyroxine C^{14}.

ually declining over remainder of the collection period. There is a slight delay in appearance and peak of activity in the lymph when compared with plasma. The amount of C^{14} thyroxine in the plasma remained higher than in the lymph throughout the 8 hour experimental period. The plasma level showed an increase of 2 to 4 times that of lymph, during the first 2 hours, after which time this difference was gradually reduced.

Fig. 3 shows concentration of thyroxine/g of plasma and lymph protein. These values were calculated on the basis of determinations of total protein content of these respective fluids at zero time (6.13 g % for plasma; 3.51 g % for lymph). This method requires some discussion. To follow the plasma and lymph concentrations of thyroxine for an 8 hour period, a fairly large amount of protein fluid was removed from the rat. Approximately 3.12 ml of whole blood was withdrawn, and the entire lymph flow in this time interval, about 5-6 ml was collected. These withdrawals were of course performed on 2 different groups of animals, because a single animal could not have sustained both hemorrhage and lymph deprivation. Because of this, it would seem that to calculate thyroxine concentration relative to initial protein levels would be invalid. According to Drinker and Field(7) however, there is a close correlation between plasma and lymph protein content. Thus, drainage of lymph will not only reduce its own protein concentration, but will effect a proportional lowering of plasma protein concentration; hemorrhage, likewise, will cause a hemodilution and thus a reduction in plasma proteins, and at the same time will induce a parallel decrease in lymph proteins. Calculation of thyroxine concentration/g of zero hour protein concentration, should reflect the relative changes that take place over a period of time, since both fluids will vary their protein concentration in a similar manner. In Fig. 3 then, the actual concentrations of thyroxine/g of protein are undoubtedly higher in the later hours than that shown, since the values are determined on the basis of initial protein content. But the relative changes in concentration remain the same. Fig. 3 indicates that after the 4th hour the lymph contains 30% more thyroxine/g of protein than does the plasma. Salter believes this is due to selective leaking of the small albumin and alpha-1 globulin molecules, the carriers of thyroxine, from the capillaries into the interstitial fluid. Salter found lymph to contain twice the amount of thyroxine as plasma when

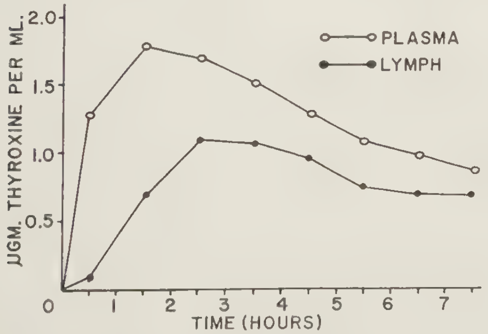

FIG. 2. Concentration of thyroxine C^{14} in plasma and lymph after a single subcut. inj. of 0.78 mg/kg.

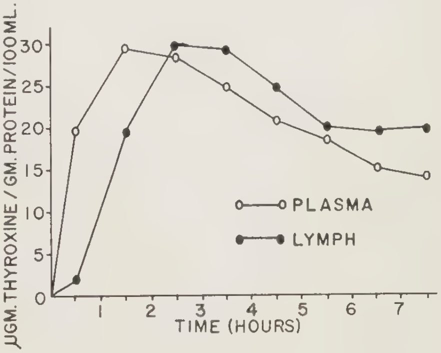

FIG. 3. Concentration of thyroxine C^{14}/g of plasma and lymph protein after a single subcut. inj. of 0.78 mg/kg.

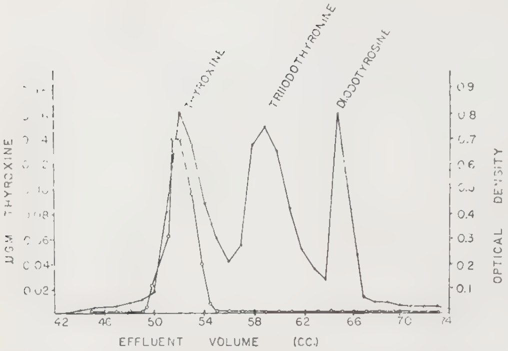

FIG. 4. Cellulose chromatographic analysis of butanol extracts of pooled (2 to 4 hr) lymph samples. (Solid dots represent known materials; open circles, material present in lymph extracts.)

calculated on a per gram protein basis; this may be due to the fact that he obtained his lymph from the cervical lymphatics, a region in which the A/G ratio is considerably higher than in thoracic duct lymph. Thoracic duct lymph has a varied origin, obtaining its fluid from the lower extremities and from abdominal organs. The liver is responsible for much of the duct's lymph, and since, according to Starling(8) the porous capillaries of the liver, unlike those of other tissues, allow all but the largest globulin molecules to pass through, the lowered A/G ratio of the thoracic duct lymph is no doubt due to the flow of beta globulin from the liver. Since thyroxine is largely associated with the albumin and alpha-1 fraction of lymph protein, the increase in beta globulin would reduce the concentration of thyroxine when expressed/g of protein. Thus, a 30% elevation of thyroxine/g of protein in duct lymph over plasma lymph would be significant, and would tend to substantiate Salter's findings.

The results of cellulose chromatographic analysis of butanol extracts of pooled lymph samples collected from 2 to 4 hours after injection are shown in Fig. 4. The radioactive peak of the lymph extracts appeared to be well within the limits of the known thyroxine and therefore identified as such. These results indicate that the butyl extractable radioactivity present in the lymph after C^{14} thyroxine administration represents unaltered thyroxine. It therefore seems likely that thyroxine, released into the circulation from the thyroid gland during this early period (2-4 hr), is bound to the smaller molecular sized protein fractions of the plasma which preferentially escape into the extravascular fluid transporting to the peripheral cell environment "hormonal iodine" in the form of unaltered thyroxine. Although no analysis of the 8 hr lymph sample was made it is possible that by this time a significant amount of administered C^{14} thyroxine could have been deiodinated by the peripheral cells and C^{14} triiodothyronine would appear in the lymph. The level of triiodothyronine in any case would remain low as it is less firmly bound to the plasma protein and readily displaced by thyroxine(9) and consequently rapidly eliminated in the urine, allowing a minimum of recirculation.

Summary. Plasma and thoracic lymph concentrations of thyroxine-l-C^{14} were followed in normal male rats after a single subcutaneous injection of 780 μg/kg body weight. Approximately 3% of administered dose appeared in the lymph during the 8 hour experimental period. The level of radioactivity in the lymph was lower than the plasma on a volume basis but significantly higher if calculated on the basis of protein content. Cellulose column chromatographic analysis of lymph samples taken between 2 and 4 hours, revealed the radioactivity present to be unaltered thyroxine.

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Development of Seizure Patterns in Newborn Animals. Significance of Brain Carbonic Anhydrase.* (23411)

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The pattern and threshold of electroshock convulsions have been investigated by Gellhorn and Ballin(1) in rats 4 weeks of age and older. With increasing age, susceptibility to seizures was diminished and, in older animals, a stronger electroshock stimulus was required in order to induce a tonic seizure pattern.

The susceptibility of young rats to experimental seizures is in accordance with the peak incidence of seizures in childhood. However, since seizures of the cryptogenic type are unusual during early infancy, and the major tonic pattern is observed rarely at this age, it seemed important to investigate further the convulsive pattern and threshold in young animals and to study susceptibility to induced seizures from the time of birth. Animal species of different degrees of maturity at birth were selected so that changes in seizure threshold which might occur with increasing age and concomitant alterations in brain chemistry could be correlated.

Carbonic anhydrase is one of a number of enzymes in the brain of the young rat which shows a sharp increase in activity during the first month of life(2). Acetazolamide (Diamox), an inhibitor of this enzyme, has an anticonvulsant effect(3), and previous observations of the mechanism of action of this drug suggest that brain carbonic anhydrase is of possible significance in the seizure process (4). In the present investigation, the relation of the enzyme to the seizure threshold has been studied further. Levels of carbonic anhydrase activity in the brain of the young rat and of the guinea pig, an animal more mature at birth, have been correlated with development of susceptibility to seizures induced by electroshock stimuli.

Methods. The young rats used in this experiment were bred from the Holtzman albino strain. Seizure patterns were studied first in rats of 3 weeks of age and were graded from 1 to 5, according to the type of response with successive increments in strength of an electroshock alternating current. The current was applied by corneal electrodes for 0.2 sec. The threshold stimulus required for maximal tonic seizures (50 m.amps.) in these animals was used subsequently for the examination of seizure patterns in rats between the ages of 1 and 35 days.

Of 10 litters of rats included in the study, 3 litters were tested only once, 3 litters twice, and 4 litters on 3 occasions. The interval between repeated electroshock stimuli was 3 to 16 days with an average of 7 days and, in agreement with the findings of Woodbury and Davenport(5) in older rats, was sufficient to permit complete recovery from the previous seizure response. The response to stronger electroshock stimuli of 100 and 200 m. amps. was observed in additional litters of rats between 4 and 24 days old. Sixteen guinea pigs in 5 age groups between 1 and 31 days were tested principally with current stimuli of 100 to 400 m.amps.

In parallel with the investigation of seizure patterns, the carbonic anhydrase activity in saline extracts of brain of rats and guinea pigs was estimated by the manometric method of Meldrum and Roughton(6). Animals were decapitated and, after removal of macroscopic blood, the cerebral cortex and subcortical white matter were weighed and homogenized with 0.9% sodium chloride solution, in a dilution of 1:20. The entire cerebral hemisphere of the young rat and a portion of the hemisphere (temporal pole) of the guinea pig brain were employed. The carbonic anhydrase activity of the supernatant fluid was determined after centrifugation. In 4 rats of different

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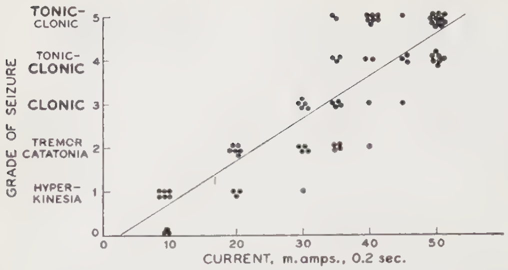


FIG. 1. Grade of electroshock seizure in 21-day-old rats in relation to strength of current stimulus. Each point represents response of one test animal.

ages, the enzyme activity in contaminating red cells was estimated and was found to be less than one per cent of that in the brain tissue specimen. The wet and dry weights of the brain were recorded for rats and guinea pigs in the age range studied.

Results. Seizure patterns. In rat 21 days old, grade of electroshock seizure was related to strength of the current stimulus as follows (Fig. 1): *grade 1,* hyperkinetic behavior sometimes associated with transient opisthotonus and breast-stroke swimming movements (10 m.amps); *grade 2,* stunning, tremor of the head, abduction of fore-limbs, and catatonic posture (20 m.amps.); *grade 3,* generalized symmetrical clonus with loss of posture (30 m.amps.); *grade 4,* transient tonic flexion and extension of the forelimbs followed by prolonged generalized clonus (40 m. amps.); *grade 5,* tonic flexion and prolonged tonic extension of both fore and hindlimbs followed by mild clonus and post-ictal depression (50 m.amps.). Similar seizure patterns in response to electroshock stimuli of different intensity have been reported in older rats(5).

Fig. 2 shows the seizure patterns of rats between 1 and 35 days of age in response to the threshold electroshock stimulus for maximal tonic seizures in 21-day-old rats (50 m. amps. applied for 0.2 sec.). In rats aged 20 to 35 days, the electroshock seizure was mainly *tonic* in pattern (grade 5); between 10 and 20 days, the *clonic* type of seizure predominated (grade 3); and from birth to 10 days, neither tonic nor symmetrical clonic seizures could be elicited with a current stimulus of 50 m.amps. The grade of response was more severe with the stronger stimuli (100 and 200 m.amps.) but, in rats aged 1 to 15 days, a maximal tonic seizure could not be elicited.

In the guinea pig, unlike the rat, there was little change in the electroshock seizure response from birth to 35 days of age (Fig. 3). The clonic response predominated and a maximal seizure with a hind-limb tonic exten-

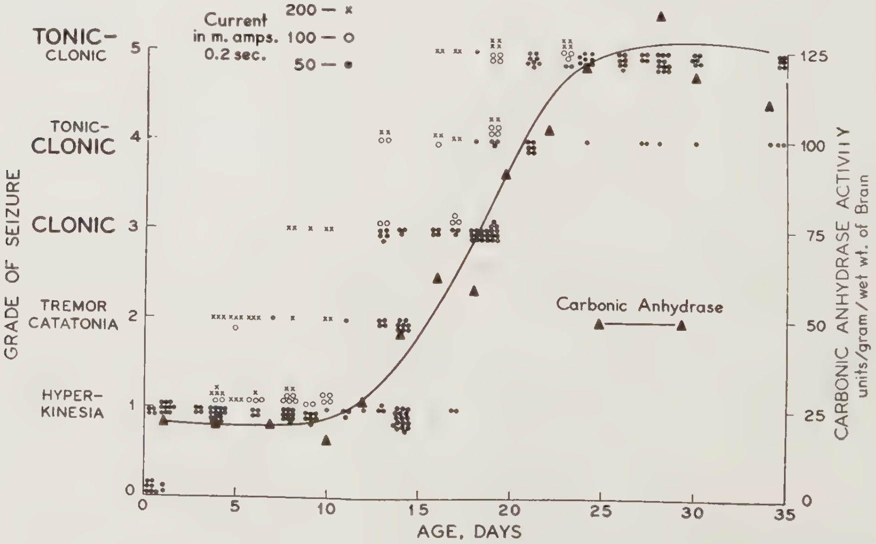


FIG. 2. Grade of electroshock seizure in young rats in relation to age and to level of brain carbonic anhydrase activity. Each point, open circle, and cross represents response of one test animal. Each triangle represents the mean of separate estimations on each of 2 to 4 animals.

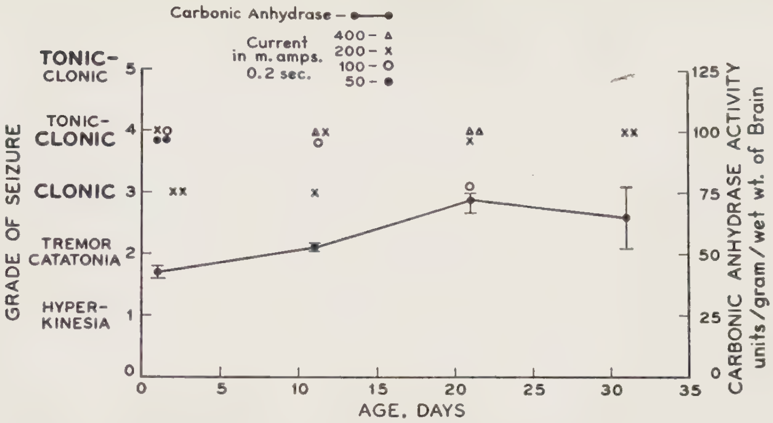
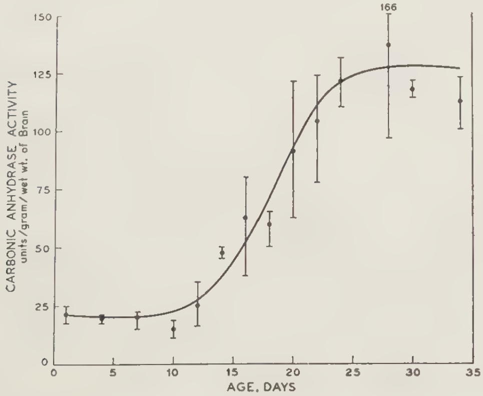


FIG. 3. Grade of electroshock seizure in young guinea pigs in relation to age and to level of brain carbonic anhydrase activity. Points and vertical lines represent respectively mean value and range of separate estimation on each of 2 or 3 specimens.

sor component could not be induced, despite the use of current stimuli of 100 to 400 m. amps.

Carbonic anhydrase determination. The carbonic anhydrase activity per unit weight of brain in relation to the age of the young rat is shown in Fig. 4. The manometric method for determination of carbonic anhydrase employed in this study yielded higher levels of enzyme activity than those obtained in a previous investigation(2); in addition, with this method carbonic anhydrase activity was demonstrated in the brain of rats at birth. A 6-fold increase in activity occurred between 10 and 25 days of age. During this interval, the water content of the brain fell from 86% to 80% (Fig. 5), and increase in enzyme activity was 4-fold when expressed as units per gram of dry brain. In contradistinction to the slow increase in body weight of the rat during this developmental period, the total brain weight rose sharply and the enzyme activity, calculated as units per whole brain, showed a 30-fold increase. In Fig. 2, the developmental pattern of brain carbonic anhydrase and the grades of electroshock seizure in the rat are superimposed.

In the guinea pig, unlike the rat, there was little change in brain carbonic anhydrase activity from birth to 31 days of age, and the



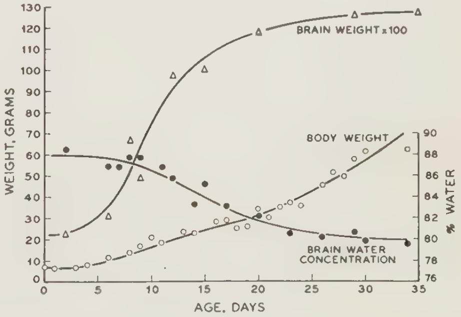


FIG. 4 (left). Development of carbonic anhydrase activity in the brain of the young rat. Points are mean values and vertical lines represent range of separate estimations on each of 2 to 4 animals.

FIG. 5 (right). Body wt, total brain wt, and brain water concentration in relation to age of young rats. ○, mean wt of 12 rats. △, mean wt of 5 brains. ●, mean water conc. of 3 brains.

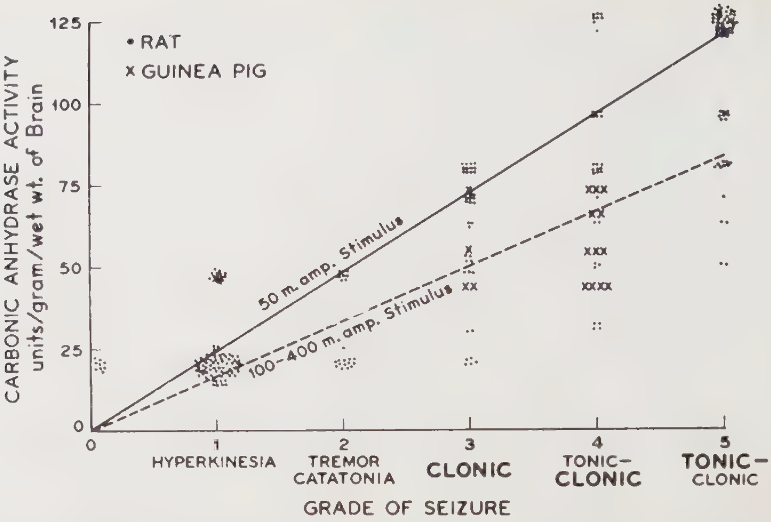


FIG. 6. Grade of electroshock seizure in rats and guinea pigs aged 1 to 35 days in relation to brain carbonic anhydrase activity. Continuous and broken diagonal lines indicate respectively the relationships obtained with currents of weak and strong intensity. Guinea pigs tested principally with stronger stimuli.

variation in electroshock seizure response remained correspondingly small during this age period (Fig. 3). In addition, there was no significant change in total weight and water content of the brain, but a 3-fold increase in body weight was observed. The degree of maturation of the guinea pig corresponded with that of the 15- to 20-day-old rat, both with regard to the grade of seizure response and to the level of brain carbonic anhydrase.

Discussion. A close correlation between the developmental pattern of brain carbonic anhydrase and the grade of seizure in response to electroshock has been demonstrated in 2 animal species of different degrees of maturity at birth. When the brain enzyme activity in rats and guinea pigs, in the age range tested, is plotted against the grade of seizure in response to stimuli of fixed intensity, a linear relationship is observed of the same order of magnitude in both animal species (Fig. 6). Biochemical maturation and susceptibility to seizures were directly related to total weight and solid constituents of the brain but not to body weight. In the young guinea pig, an increase in body weight occurred without significant alteration of the seizure threshold and, in the rat between 10 and 25 days of age, a slow increase in body weight was associated with a rapid fall in the seizure threshold. In older rats, however, Davenport(7) found that the electroshock threshold was directly related to the body weight.

The inability of the newborn rat to respond maximally with a tonic electroshock *seizure pattern* is in accordance with clinical observations and the infrequent occurrence of generalized tonic-clonic seizures in immature infants. The present findings agree also with the report by Grossman(8), of an absence of "tonic" or fast spike discharge in response to electrical stimulation of the cortex of immature kittens. The high *seizure threshold* in the newborn rat corresponds with the low susceptibility of the young infant to convulsions associated with fever and to seizures of the cryptogenic type.

In previous work(4), it has been shown that the anticonvulsant action of acetazolamide (Diamox)[†] in mice is directly related to inhibition of brain carbonic anhydrase activity. That this enzyme is of significance in the seizure process is suggested by this study and is supported further by the present demonstration of a relation between the susceptibility to induced seizures and the level of carbonic anhydrase activity in the brain. Preliminary studies of the effect of Diamox on electroshock seizures in young rats have shown a specific activity against the maximal seizure response. While the *tonic* type of seizure exhibited by the older rats was abolished by very small doses of the carbonic anhydrase inhibitor, the *clonic* pattern was controlled only by large doses, and the hyperkinetic behavior of 10-day-old rats was refractory to the drug.‡ Similar results were obtained with Diamox in infants and children with grand mal and generalized myoclonic seizures; the tonic seizure was controlled more effectively than the myoclonic pattern(9).

[†] Diamox was kindly supplied by Dr. James D. Gallagher, Lederle Medical Research Department, American Cyanamid Co.

These observations suggest that brain carbonic anhydrase is of functional significance in development of susceptibility to maximal *tonic* convulsions, in which a generalized spread of the seizure discharge is probably involved. The significance of this enzyme in the mechanism of clonic seizure patterns is less well defined and, on the basis of present evidence, the following possibilities are suggested. Such seizures, which are minor in character and which probably involve focal neuronal discharge, may be unrelated to the activity of brain carbonic anhydrase. As the result of observations in the rabbit of the effects of carbonic anhydrase inhibition by thiophene-2-sulfonamide, Davenport(10) has concluded that, when stimulated locally, the electrical activity of the cerebral cortex is independent of carbonic anhydrase; and the resistance to Diamox of the minor convulsive activity observed in the newborn rat would lend support to this conclusion.

Alternatively, an impairment of diffusion of the drug might explain the relatively low anticonvulsant potency of Diamox in the minor hyperkinetic and clonic seizure patterns of the newborn rat, when compared with its activity against tonic seizures in the older rat. The potency of brain enzyme inhibition *in vivo* may be lower than that expected by *in vitro* tests(4). The increase in brain carbonic anhydrase activity with age corresponds temporally with the rapid increase in weight and in solid constituents of the brain. The greater anticonvulsant potency of the enzyme inhibitor observed in older animals might then be associated with changes in cerebral localization of the enzyme and possibly, with facilitation of diffusion of the drug. The possible role of brain carbonic anhydrase in development of susceptibility to minor or clonic seizures remains to be clarified.

Summary. Electroshock seizures have been studied in young rats and guinea pigs, and a correlation has been demonstrated between the developmental pattern and threshold of the induced seizures and the level of carbonic anhydrase activity in the brain. In young rats aged 20 to 35 days, the seizures were mainly tonic in pattern, whereas the clonic type of seizure predominated in rats aged 10 to 20 days. Failure to induce convulsions in the newborn rat was associated with a low level of carbonic anhydrase activity in the brain. The degree of maturation of the newborn guinea pig was equivalent to that of the 15-day-old rat, both with regard to the type of seizure response and the level of brain carbonic anhydrase activity. That brain carbonic anhydrase is of functional significance in the generalized spread of the seizure discharge and in the production of a maximal tonic convulsion is suggested by these data and by observations of the anticonvulsant activity of acetazolamide.

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A Radioisotope Technic for Determining Allantoic Fluid Volume of Embryonated Eggs.* (23412)

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Romanoff *et al.*(1,2) have published curves showing that the volume of allantoic fluid in a fertile chicken egg increases through the thirteenth day of incubation, then decreases through the remainder of embryonic development. Calculation of the amounts of waste products excreted by chick embryos requires in addition to the concentration figures, a knowledge of the allantoic fluid volume of fertile eggs at progressive stages of incubation. Embryonated eggs have been used extensively in this laboratory in studying experimental porphyria(3,4,5). Early in the course of these studies it was observed that porphyric eggs appeared to contain somewhat larger volumes of allantoic fluid than their controls several days after injection of the porphyria-producing drug. Thus, in order to evaluate the porphyrin outputs of porphyric chick embryos with a reasonable degree of accuracy it was necessary to determine volume of allantoic fluid present in eggs treated in this manner. Furthermore, it seemed advisable to determine in the same manner the allantoic fluid volume of normal embryos subjected to the conditions used in these experiments. For this purpose, a dilution technic, utilizing radioiodinated human serum albumin (RISA)® has been developed, and the results using this technic are reported here.

Materials and method. 1. *Radioassay*: Both gas-flow and well-type scintillation detectors have been used in this work. Instruments used were the windowless pre-flush flow counter (Mark 12 Model 3), manufactured by Radiation Counter Laboratories (well size 11/32 x 2″) attached to a Nuclear Super-scaler, Model 172, and the Nuclear Model DS-3 scintillation counter attached to a Berkeley Model 2020 scaler. 2. *Eggs*: Pure strain White Leghorn eggs, obtained from a local hatchery after a week's incubation, were maintained in a forced-draft incubator at 37.5°C and 55-60% relative humidity. In this laboratory, it was found convenient to designate incubation age as follows: 24-48 hours incubation, one day old; 48-72 hours incubation, 2 days old, etc. Under this system, the egg is designated as up to one day younger than in Romanoff's studies(1,2). 3. *Experimental Porphyria*: Hepatic porphyria was induced by injecting 10.0 mg of allylisopropylacetylcarbamide (Sedormid)®(3) or 5.0 mg of allylisopropylacetamide (AIA)(5) in a volume of 0.5 ml into the yolk sacs of 8-day embryonated eggs. Control eggs received 0.5 ml of the drug vehicle at the same time. 4. *RISA*: For injection, RISA was diluted with 0.9% NaCl to give an activity of approximately 0.1 μc/ml. A volume of 0.10 ml of this diluted solution was injected into the allantoic sac as described below. The amount of radioactivity injected was determined by preparing 4 standard samples which were counted each day at the same time as the allantoic fluid samples. For the gas-flow counter, counting standards were prepared by further diluting the injected solution 1:5 with physiological saline, plating 0.10 ml of the second dilution in the center of a 2″ aluminum planchet and drying under an infra-red lamp. Counting standards for the scintillation detector were prepared by diluting 0.10 ml of the solution prepared for injection with 1.90 ml of 0.9% saline in ½ x 5 inch test tubes. The tubes were stoppered with Parafilm-wrapped corks and stored in the refrigerator until used. 5. *Experimental procedure*: Determinations were begun with 8-day embryos. Each egg was candled, its air sac outlined with

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pencil, and the position of the embryo marked. RISA was injected into the allantoic sac through a hole drilled in the shell just above the margin of the air sac and above the embryo. Injection was made from a Krogh pipette set at 0.10 ml bearing a ½″ 25 gauge hypodermic needle, the needle being inserted straight down through the hole in the shell. The eggs were then returned to the incubator during the time allowed for mixing. In some experiments, samples were withdrawn after ½ hour and again after 1 hour in the incubator. In others, samples were withdrawn after 1 hour in the incubator and again after 3 hours in the refrigerator (4 hours after RISA). Samples were withdrawn through a different hole in the top of the egg (the hole used for injecting via the yolk sac was usually used) with a 20 gauge, 1″ or 1¼″ needle, perforated in the terminal centimeter as described by Green and Freymann(6), and attached to a 1.0 ml tuberculin syringe. For the gas-flow counter, 0.20 ml of the fluid was plated in the center of a 2″ aluminum planchet, and dried under an infra-red lamp. Samples were prepared for the scintillation counter by transferring 0.20 ml of allantoic fluid to a ½″ x 5″ test tube and diluting with 1.80 ml of 0.9% NaCl. If the activity of such samples was too low, a 0.50 ml aliquot of allantoic fluid diluted with 1.50 ml of 0.9% NaCl was used. Measurement of these aliquots of allantoic fluid with the tuberculin syringes used to withdraw them proved accurate and convenient. On several occasions, 0.20 ml samples of yolk, amniotic fluid, and embryo homogenate (single embryos homogenized in water, then brought to 10 ml) were plated and counted to check for possible loss of RISA from the allantoic sac during mixing. To check the effect of allantoic fluid on the counting rate of RISA, an aliquot of the RISA solution injected was diluted 1:5 with allantoic fluid from normal 14 day embryos; then four 0.10 ml samples of this solution were plated, dried, and counted in the same manner as the counting standards diluted with saline. 6. *Calculations*: The number of counts injected was calculated from the average number of counts above background (C/M) of the 4 counting standards prepared as outlined above.

With gas-flow counter: C/M injected = Average C/M of standards × 5

With scintillation counter: C/M injected = Average C/M of standards

With both types of detector, using 0.20 ml samples of allantoic fluid for radioassay:

$$\text{Allantoic fluid volume} = \frac{\text{C/M injected}}{\text{C/M/ml}} = \text{ml.}$$

Results. 1. *Radioactivity in the embryo and embryonic fluids.* Amniotic fluid, yolk, and an homogenate of the embryo were checked for radioactivity 1 hour after the injection of RISA via the allantoic sac on 4 different days. No count exceeded background, thus excluding a loss of RISA from the allantoic sac. 2. *Effect of allantoic fluid on the counting rate of RISA*: Diluting the RISA used for injection with allantoic fluid rather than saline failed to alter the counting rate. (Sample diluted with saline = 3990 C/M; sample diluted with allantoic fluid = 4010 C/M.) 3. *Time required for adequate mixing*: The volumes calculated after allowing only ½ hour for mixing differed from those calculated after 1 hour's mixing by an average of 0.82 ml, while the volumes calculated after 4 hours' mixing varied from the 1 hour figure by an average of only 0.24 ml. Thus, it appears that ½ hour was insufficient for thorough mixing of RISA with allantoic fluid. 4. *Allantoic fluid volume of normal and porphyric chick embryos*: These data are summarized in Table I. To arrive at these figures the average of determinations carried out on the same egg after 1 and 4 hours' mixing was used as the allantoic fluid volume of that egg. Where 2 values were not obtained, 1 hour was allowed for mixing. Volumes calculated after only ½ hour's mixing were ignored. In both normal and porphyric embryos, allantoic fluid volume increased progressively up to 12 days of age, then steadily decreased. Four days after injection of the porphyria-producing drug (12-day embryos), porphyric embryos displayed a considerably larger allantoic fluid volume than the controls and this relationship persisted throughout the period studied. The large standard deviations emphasize the variability of this quantity.

TABLE I. Allantoic Fluid Volume of Normal and Porphyric Chick Embryos at Progressive Stages of Embryonic Development.

Incubation age (days)	Days after drug treatment	Avg vol (ml)*	
		Control	Porphyric
8	0	2.64 ± .43 (18)	—
9	1	4.25 ± 1.31 (17)	3.57 ± .96 (21)
10	2	5.56 ± 1.43 (18)	5.00 ± 1.02 (22)
11	3	5.78 ± 1.10 (16)	6.65 ± 1.57 (21)
12	4	7.01 ± 2.26 (16)	8.60 ± 2.40 (20)
13	5	6.99 ± 1.18 (15)	8.37 ± 1.77 (20)
14	6	6.45 ± 1.16 (14)	7.50 ± 1.68 (19)
15	7	5.66 ± 1.17 (17)	6.56 ± 1.64 (20)
16	8	4.62 ± 1.53 (17)	5.43 ± 1.42 (20)
17	9	3.72 ± 1.11 (12)	4.65 ± 1.73 (13)

* Figures in parentheses indicate No. of observations from which avg value was derived.

Discussion. When the average values are plotted, both sets of data give smooth curves (Fig. 1) except for the value for 11-day normal embryos. However, if this figure be ignored, the curve outlined by the other 9 points, closely resembles the allantoic fluid volume curve presented by Romanoff(2). As pointed out above, these volumes vary widely at a given time. In view of this great variability, it seems possible that the number of eggs used (16) to obtain this value was not large enough, statistically, to provide a really good estimate of the average. None of Romanoff's curves(1,2) exhibit a break at this point and his data were obtained from hundreds of eggs. Thus, it seems reasonable to read the allantoic fluid volume (6.45 ml) for this point from the curve outlined by the other 9 points.

In these studies, maximum allantoic fluid volume was found to occur at 12 days incubation age by the convention adopted in this laboratory, or 13 days by Romanoff's system. This coincides exactly with Romanoff's observations(1,2). The average maximum volume of 7.01 ml for normal embryos reported here agrees well with the value of approximately 6.5 ml reported by Romanoff(2) especially when the 0.5 ml of physiological saline injected into the yolk sacs of the control embryos is taken into account.

Retarded growth of porphyric embryos has been reported previously(3). The observation of a considerably larger volume of allantoic fluid in porphyric eggs 4 days after injection of the porphyria-producing drug coincides with(7) or just precedes(3) the appearance of a statistically significant difference in the weights of porphyric and control embryos. Elimination of metabolites which they are unable to utilize for growth probably accounts for the increased volume of allantoic fluid found in porphyric eggs. Differences between allantoic fluid volumes of normal and porphyric embryos were not significant statistically, but this probably resulted from too few observations on a highly variable figure. Gross observations on a much larger number of eggs leave little doubt that the allantoic fluid volumes of porphyric eggs consistently deviate from normal in the manner indicated.

Summary. A method for determining the

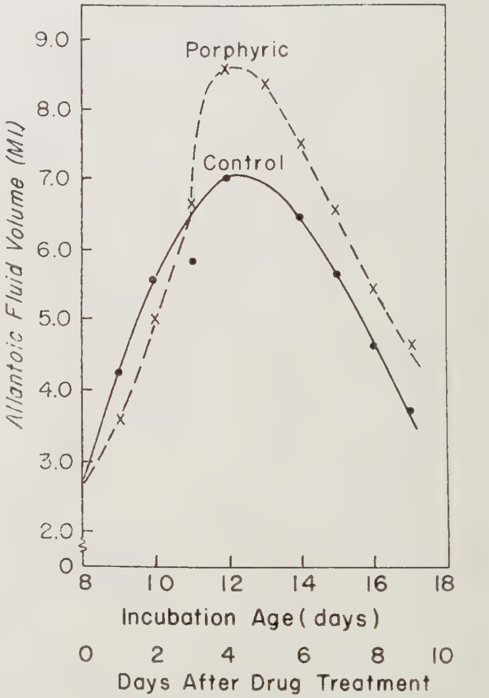

FIG. 1. Allantoic fluid vol of normal and porphyric chick embryos.

allantoic fluid volume of embryonated eggs by dilution of RISA is described. Experimental porphyria in chick embryos results in a marked increase in allantoic fluid volume.

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Studies on Propagation of the Col SK Group of Viruses in Various Tissue Culture Media.* (23413)

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Cultivation of Col SK virus *in vitro* on various cell substrates began with the early work of Jungeblut and Sanders(1,2). Explants of minced embryonic tissues, suspended under controlled oxygen-carbon dioxide tension in ox serum ultrafiltrate, were used for this purpose. The virus multiplied, to varying extent, on brain from mice and guinea pigs or on whole chick material; non-nervous embryonic tissue also supported a basic level of neurotropic virus growth. The virus yield was approximately the same from cells as from culture fluid. While the procedure did not permit demonstration of cytopathogenic effects, Huang(3) discovered that there was less glycolysis and acid production in the infected medium than in non-infected controls. The above results were confirmed by Shean and Schultz(4) who studied the mouse explant requirements of Col SK and MM virus in comparison with those of Theiler's GD VII and FA virus. Growth of MM virus in various extraneural tissues of mice or hamsters was later reported by Chambers, Smith and Evans(5,6), by Evans and Chambers(7) and by Fabiyi(8).

Only sporadic attempts have been made to grow viruses of the Col SK group in tissue cultures of embryonic or adult cells from man or monkey, utilizing modern methods of tissue cultivation. Col SK virus has been reported as growing on cynomolgus monkey testicular cell cultures(9) but the virus apparently does not multiply on cynomolgus monkey kidney (10); growth of MM virus has also been obtained in cultures of testicular cells from rhesus monkeys(11) or from man(6) and, in addition, on human fetal fibroblasts(12). Barski and Lamy(13) reported that Mengo virus multiplies abundantly in tissue cultures of kynocephalus monkey kidney cells, inducing characteristic cytopathogenic effects after 2 days and causing complete destruction of the culture within 4 days; the virus also grew on embryonic human kidney tissue.

Little is known about growth of viruses of the Col SK group on tumor cells. Among malignant mouse cell cultures, L (Earl) cells are said to support non-cytopathogenic growth of EMC virus(14), but Col SK virus failed to multiply on 2 transplantable mouse carcinoma or sarcoma cells studied by Kret (9). On the other hand, mice bearing the Ehrlich ascites tumor are apparently more susceptible to infection with Mengo virus than are normal mice(15); growth of EMC virus in Ehrlich ascites tumor transplants, with marked oncolysis, was reported by Levy and Snellbaker(16). Quite recently, multiplication, with cytopathogenic effects, was described for EMC virus by Eagle *et al.*(17) in KB cell tissue cultures, a human epidermoid carcinoma of the floor of the mouth.

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The above review suggests that there may be certain differences in growth requirements of the several strains of the Col SK group on fibroblastic or epithelial cell substrates. To provide more information on the subject we have investigated comparatively the cellular responses of L cell, HeLa cell and KB cell tissue cultures to infection with Col SK, MM, F, EMC and Mengo virus.

Materials and methods. The Col SK, MM and F strains were available in this laboratory as mouse brain passage virus. The EMC strain (rat brain passage) and the Mengo strain (mouse brain passage) were kindly supplied by Dr. Joel Warren; we are also indebted to Dr. K. Habel for sending us a sample of the EMC strain grown in L cell tissue culture. L cells were obtained, through the courtesy of Dr. M. Holden, from Dr. Earl. HeLa cells were generously supplied by Dr. J. Syverton. We owe thanks to Dr. H. Eagle and to Dr. B. Mandel for providing us with KB cells. *L cells* were grown, at 37°C, in 200 cc screw-cap square bottles in a medium consisting of 40% horse serum (supplied by the New York City Health Dept.), 40% Hanks balanced salt solution and 20% chick embryo extract, plus antibiotics (200 units penicillin and 100 γ streptomycin per cc). After 7-10 days growth, the cells were trypsinized (0.5% Difco trypsin in Hanks solution for 30 minutes at 37°C) and suspended (200,000 cells per cc) in Hanks solution containing 20% horse serum and 10% chick embryo extract. *HeLa cells* were grown in bottles in a medium consisting of 20% horse serum, 10% human serum and 70% Hanks solution containing 0.1% yeast extract. After 4 days growth the cells were trypsinized and suspended (100,000 cells per cc) in Scherer's maintenance solution containing 10% horse serum. *KB cells* were grown in bottles in Hanks solution containing 10% horse serum, 0.5 cc lactalbumin hydrolysate and 0.1% yeast extract. After 4 days growth the cells were trypsinized and suspended (100,000 cells per cc) in Scherer's maintenance solution with 10% horse serum. All cell suspensions were then distributed in 0.5 ml volumes into small rubber-stoppered stationary tubes and 2-day-old sheets of cells, after replacing the medium, were ready for infection.

The procedures employed gave excellent growth of all cell lines and the sheets could be maintained in tubes for at least 10 days in good condition. The virus inoculum usually consisted of 0.1 cc of various dilutions which was added to a volume of 0.4 cc fluid in each tube. The infected tubes were examined daily for cytopathogenic effects over a period of from 5 to 7 days. Positive tubes were harvested at the peak of cytopathogenicity and the culture fluid was transferred to new tissue culture tubes for a variable number of serial passages; potency of the virus was determined by titration in tissue culture or in mice.

Results. It was found that EMC and Mengo virus grew well on L cells, HeLa cells and KB cells; F virus multiplied only on L cells. None of the 3 substrates supported any growth of Col SK or of MM virus despite repeated attempts to adapt these strains by serial blind passage of either culture fluid or of cells; in fact, Col SK virus was lost, as a mouse-pathogenic agent, between the second and third L or HeLa cell culture transfer. Equally unsuccessful were efforts to obtain multiplication of Col SK virus on HeLa cell cultures which had been pre-treated with hyaluronidase. By contrast, virus titers of considerable height, with full cytopathogenic effects, were realized with EMC, F and Mengo virus on L cells and with EMC and Mengo virus on KB and HeLa cells (Table I).

More detailed experiments were carried out with EMC virus grown on L cells or on HeLa cells. Since transfers were made with relatively high dilutions (10^{-3}) of tissue culture fluid, it may be estimated that the original inoculum had been eliminated after the first 3 serial passages. The virus in initial passages reached a potency of between 10^3 and 10^4 TC ID_{50} per 0.1 cc culture fluid and this titer was maintained over 12 subsequent serial passages; however, comparison of infectivity in tissue culture and in mice indicated that EMC virus grown for 10 serial passages in HeLa tissue culture had suffered a marked loss of its mouse-pathogenicity (Table II).

TABLE I. Growth of Several Strains of the Col SK Virus Group in Various Tissue Culture Substrates.

	L cells					HeLa cells					KB cells				
	Inoculum					Inoculum					Inoculum				
Virus strain source	1st passage	Subsequent passages	No. of passages carried	Cyto-pathogenicity	Virus titer* TC ID_{50} (per 0.1 cc)	1st passage	Subsequent passages	No. of passages carried	Cyto-pathogenicity	Virus titer* TC ID_{50} (per 0.1 cc)	1st passage	Subsequent passages	No. of passages carried	Cyto-pathogenicity	Virus titer* TC ID_{50} (per 0.1 cc)
Col SK 610th mouse passage	10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	4	—	0	10^{-1} 10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	7	—	0	10^{-2}	10^{-1}	2	—	0
MM 260th mouse passage	10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	3	—	0	10^{-1} 10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	2	—	0	10^{-2}	10^{-1}	2	—	0
F 25th mouse passage	10^{-2}	10^{-1} 10^{-2} 10^{-3}	4	+	10^{-2}	10^{-1} 10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	4	—	0	10^{-2}	10^{-1}	2	—	0
Mengo 25th mouse passage	10^{-2} 10^{-3}	10^{-1} 10^{-2} 10^{-3}	4	+	$>10^{-3}$	10^{-2}	10^{-1} 10^{-2} 10^{-3}	3	+	10^{-2}	10^{-2}	10^{-1} 10^{-2}	3	+	10^{-3}
EMC 15th rat passage	10^{-2} 10^{-3}	10^{-1} 10^{-2} 10^{-3}	2	+	10^{-2}	10^{-2}	10^{-1} 10^{-2} 10^{-3}	3	+	10^{-3}					
EMC L cell passage	undil. 10^{-1} 10^{-2}	10^{-1} 10^{-2} 10^{-3}	5	+	$10^{-4.5}$	undil. 10^{-1} 10^{-2}	10^{-1} 10^{-2} 10^{-3}	12	+	$10^{-4.5}$	undil.	10^{-1} 10^{-2}	3	+	10^{-3}

* Harvest from last passage indicated.

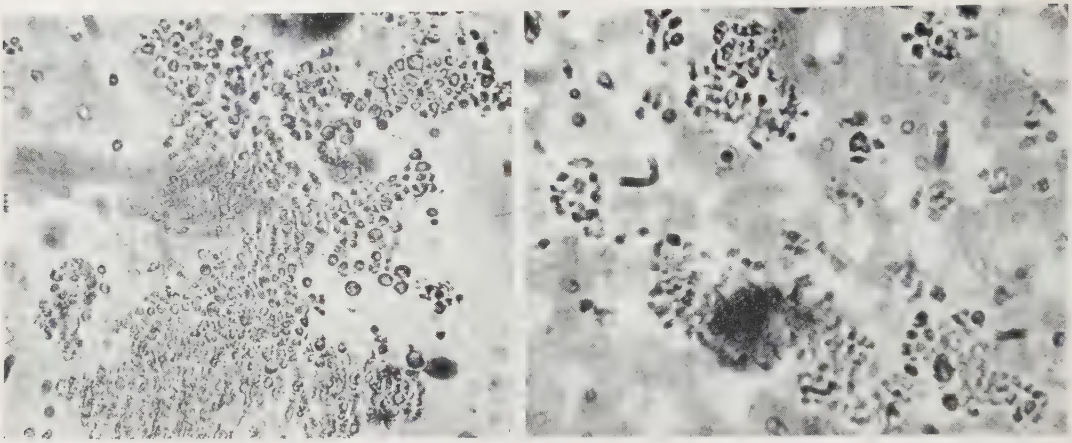


FIG. 1. *Left*—L cell culture infected with L EMC tissue culture virus (V passage). *Right*—HeLa cell culture infected with HeLa EMC tissue culture virus (V passage).

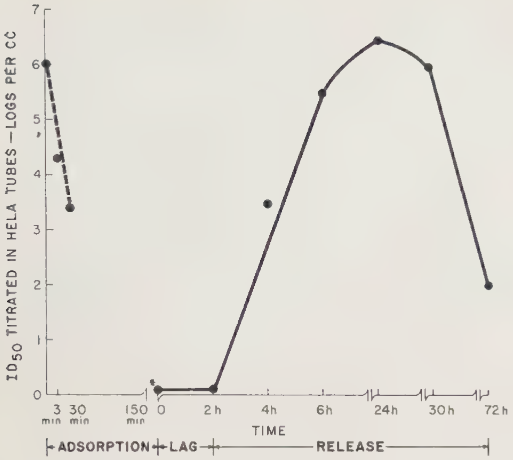
The virus grew to maximum titer in L and in HeLa cell culture tubes within 1-4 days, depending on size of inoculum, and cytopathogenicity became evident within 18-72 hours after inoculation of the sheets. In the early stages, individual cells showed rounding up with increased granularity; later extensive disintegration of the entire cell layer occurred, followed occasionally by detachment of the sheet. While similar in some respects, the over-all picture differed clearly from that produced by the poliomyelitis viruses on HeLa cells. The cytopathogenic effects of EMC virus, in its fifth serial tissue culture passage on L or HeLa cells, are illustrated in Fig. 1.

The process of adsorption and of subsequent multiplication of EMC virus on HeLa cells was studied more closely in the following experiment: A layer of approximately 1 million HeLa cells, on the 7th day of its growth in a bottle, was washed 3 times with Hanks solution and the medium was replaced with 9 cc of maintenance solution containing 10% horse serum. The bottle was then infected with 1 cc undiluted EMC virus tissue culture fluid (IV HeLa bottle passage), resulting in a 10-fold dilution of the virus. The seed virus had a potency of about 10^5 TC ID_{50} per 0.1 cc of fluid providing a ratio of 1:1 between number of susceptible cells and number of infectious particles. Immediately after inoculation a sample of 0.2 cc was withdrawn from the bottle and the lost fluid replaced by 0.2 cc of medium; the bottle was then incubated and another sample of 0.2 cc was withdrawn after 30 minutes, with replacement of the medium. After an interval of 2½ hours from the beginning of the experiment the fluid of the bottle was drained, the cell layer was washed 3 times with Hanks solution and covered with 10 cc of fresh medium, and the bottle was incubated again. Considering this time of the experiment as the zero point of multiplication with subsequent release of active virus particles, samples of 0.2 cc each were removed after 2, 4, 6, 24, 30 and 72 hours, replacing the lost fluid at each interval with 0.2 cc of medium. All samples were then titrated in HeLa tubes and the virus titers expressed in ID_{50} doses per 1 cc of fluid. The data are shown graphically in Fig. 2. It is clear that there occurs in the culture fluid an almost instantaneous loss of nearly 2 logs of virus upon first contact with cells, followed by a further reduction of an-

TABLE II. Comparison of Infectivity of EMC HeLa Tissue Culture Virus for HeLa Cells and for Mice.

		Infectivity in albino mice (10-12 g)	
HeLa passage	Infectivity in HeLa TC ID_{50} (per .1 cc fluid)	ID_{50} intracer. test	ID_{50} intraper. test
I	$10^{-3.5}$	10^{-9}	10^{-8}
XI	$10^{-4.5}$	10^{-6}*	10^{-6}

* Some mice developed paralysis after greatly prolonged incubation periods (10-14 days) following inj. with 10^{-7} dilution.



*Sheet was washed three times with Hanks solution, then covered with fresh medium

FIG. 2. One step growth curve of EMC tissue culture virus in a bottle of HeLa cells.

other log of virus within the next half hour. Counting from the zero point of virus growth, evidence of multiplication becomes apparent, after a lag period of at least 2 hours, at the 4 hour interval; thereafter the amount of released virus increases rapidly over the next 2 hours, reaching a peak of 6.5 logs at 24 hours incubation. The increments in virus release were in good agreement with synchronous observations on the appearance of cytopathogenic changes over the same period of time. Once the acme of growth had been attained, early deterioration of the virus sets in and the titer drops precipitously to 10^{-2} at the 3-day interval. A similar lability of the virus is noted in making dilutions of infected culture fluids which must be kept on ice to avoid loss of potency. The curve given in Fig. 2 indicates that the entire cycle of virus adsorption, multiplication, and decay in tissue culture is extraordinarily fast, duplicating what is known of the progress of infection in susceptible animals.

To study the formation of hemagglutinin in the tissue culture medium, EMC virus was grown for several consecutive passages on HeLa cell sheets covered with a medium containing 10% horse serum which had previously been absorbed with human O and sheep erythrocytes so as to remove the natural agglutinin for these cells. Hemagglutination tests, using human O red cells as well as sheep red cells, were then carried out with tissue culture fluid harvested at various intervals after infection. The technic employed in these tests was similar to that previously described for hemagglutination tests with mouse brain virus(18), utilizing K veronal buffer as diluent for culture fluid and red cells. As will be seen from Table III, hemagglutinin—at maximum titer of between 1:2 and 1:4—appeared in the culture fluid after 24 hours incubation, with a rapid decline setting in at later intervals. In other words, formation of hemagglutinin paralleled roughly release and decay of infectious virus particles, except for a slight delay during the first 8 hours after infection. The ratio between hemagglutinin titer in tissue culture (1:2) and that of infected mouse brain (1:640) approximately agrees with the proportional difference in infectivity titers of tissue culture virus ($10^{-5.5}$) and of mouse virus (10^{-8}-10^{-9}). Hence there is good evidence that the hemagglutinin of EMC virus is actually identical with the infectious particle. This conclusion is further supported by the fact that HeLa cell tissue culture fluid, in its third serial transfer following inoculation with Col SK virus, failed to hemagglutinate and to infect mice. Therefore, there is no reason to assume that Col SK virus may have a precursor, or incomplete form which is non-infectious but which hemagglutinates.

Identification, by serological methods, of the EMC tissue culture virus grown on HeLa

TABLE III. Hemagglutinin Formation by EMC Virus Grown in HeLa Tissue Cultures.

HeLa passage	Interval since infection of culture	Hemagglutinin titer Human O cells	Hemagglutinin titer Sheep cells
I	3 days	1:4	
II	3 "	1:4	1:2
III	3 "	1:4	1:2
IV	8 hr	0	
"	24 "	1:4	
"	72 "	1:2–1:4	
"	96 "	0	
Control:	uninoculated medium	0	0
EMC virus mouse brain		1:320–1:640	1:320–1:640

cells was readily accomplished by means of neutralization and hemagglutination-inhibition tests, using a Col SK immune serum and immune sera against the 3 prototypes of poliomyelitis virus; we are indebted to Dr. H. Wenner for making available to us the poliomyelitis typing sera. Specific neutralization as well as hemagglutination-inhibition occurred with infected tissue culture fluids harvested from remote passages, (IV, VIII) and the antiviral potency of the Col SK antiserum used in these tests compared closely with that observed in similar tests with the parent EMC mouse-passage virus. Thus, the neutralization index of Col SK serum was 1:1000 as determined against 100 ID TC_{50} of virus in HeLa tissue culture or against 100 ID_{50} of mouse virus in mice (i.p. test); similarly, 2-4 hemagglutinating units of tissue culture or of mouse virus were completely inhibited by a 1:1000 dilution of the same serum. No neutralization or hemagglutination-inhibition was observed with the poliomyelitis sera.

Discussion. It is clear that different strains of the Col SK group of viruses have different growth requirements when tested in 3 tissue culture media containing mouse or human cells. Thus, whereas L cells, HeLa cells and KB cells supported cytopathogenic growth of EMC and Mengo virus, Col SK and MM virus failed to multiply on the same substrates, and F virus occupied an intermediate position by growing only on L cells; multiplication of EMC virus on KB cells had previously been reported by Eagle *et al.*(17). It is not unusual for individual strains belonging to the same virus group to differ in their ability to propagate in a given tissue culture medium, and the fact itself lends weight to the authenticity of the isolates. It is of interest that HeLa sheets inoculated with non-cytopathogenic Col SK virus were found fully protected against infection with cytopathogenic EMC virus when first and second tissue culture passages were employed which still yielded mouse-pathogenic Col SK virus; no such protective effects, however, could be demonstrated with subsequent serial transfers of Col SK virus which were non-pathogenic for mice. More information on interference phenomena between EMC virus and other viruses in HeLa tissue culture will be reported elsewhere.

The data dealing with the growth curve of EMC virus on HeLa cells are in harmony with similar observations established for other virus-cell systems. The process begins with almost instantaneous adsorption of large amounts of virus on the susceptible cell and is followed, after a short eclipse period, by a rapid increase of infectious virus particles which are released from the injured cell into the ambient fluid. Of special interest is the fact that HeLa cells are also capable of generating viral hemagglutinin, in proportion with the number of infectious units. Heretofore, hemagglutinin production by viruses of the Col SK group in infected animals has been observed only in nervous, and not in extraneural tissues(19). The definite cytopathogenic action and hemagglutinin production of EMC virus in HeLa tissue cultures offer new possibilities for the application of relatively simple serological *in vitro* methods to epidemiological surveys of infections caused by this group of viral agents.

Summary and conclusions. Results of a comparative study of the cell responses induced by several strains of the Col SK group of viruses in various tissue culture substrates are reported. L cells, HeLa cells and KB cells supported cytopathogenic growth of EMC and of Mengo virus, F virus grew only on L cells, and Col SK and MM viruses failed to grow in any of the 3 substrates. HeLa tissue cultures inoculated with EMC virus, in remote serial passage, were infectious for HeLa cells and for mice at an ID_{50} of about 10^5 per 1 cc of culture fluid and had a hemagglutinating titer of between 1:2 and 1:4. Data permitting the establishment of a growth curve of EMC tissue culture virus on HeLa cells are submitted.

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Induced Tolerance and "Homologous Disease" in X-Irradiated Mice Protected with Homologous Bone Marrow.[*] (23414)

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Several species of mammals can be protected against otherwise lethal doses of whole body X-irradiation by post-irradiation transfusion of bone marrow or spleen. Protection is associated with rapid recovery of the otherwise depleted or destroyed hematopoietic tissues as a result of reseeding and repopulation with cells of the transfused marrow, even if the marrow is of homologous or heterologous origin(1-6).[†] Mice protected against otherwise lethal doses of irradiation with homologous marrow are thereby rendered tolerant of skin grafts as well as hematopoietic tissue of the marrow donor antigenic type(7,8). Such tolerance of homologous skin and hematopoietic tissue can be abolished by introduction into the tolerant mouse of isologous lymph node or spleen cells from an unirradiated mouse of the same strain(9). The closeness of genetic relationship of the marrow donor and irradiated recipient has a great effect upon both degree of initial irradiation protection and duration of survival(8,10). In otherwise lethally irradiated mice, marrow from guinea pigs or rabbits conferred insignificant protection(8). Rat marrow gave significant but short-term protection. Any type of mouse marrow gave excellent 21-day protection, beyond which isologous marrow gave permanent protection but foreign-strain mouse marrow gave significant late mortality characteristically between the 21st or 30th day and the 60th day post-irradiation. Late mortality was more severe when the foreign strain marrow came from a donor of a different Histocompatibility - 2 genotype (A into C_{57}, Dba into A, A into CBA) than when it came from a donor of the same H-2 genotype (C_3H into CBA) as the recipient. With homologous marrow from an F_1 hybrid into the irradiated parent strain, 2 groups showed excellent protection as late as 100 days post-irradiation, comparable to that obtained with isologous marrow. Two additional groups showed significant mortality, both early and late, attributable in part at least, to the use of very young mice as irradiated recipients.

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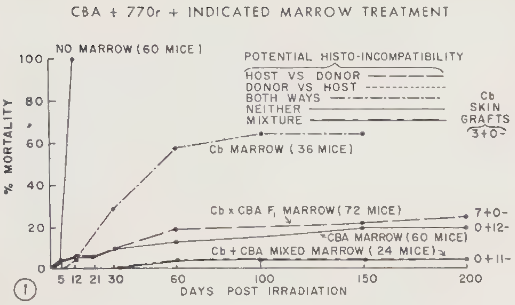
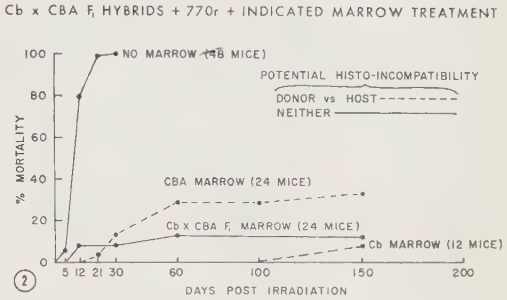
[†] Autologous—from same individual; isologous—from another individual of same inbred strain (presumably same genotype) or an identical twin; homologous from an individual of different genotype but of same species; heterologous—from a different species.

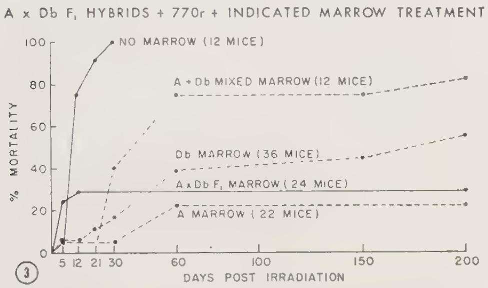
The late mortality (21 to 60 days) following good early protection with homologous marrow was preceded by progressive loss of body weight. That it was not due to recovery of the host's ability to reject the homologous hematopoietic tissue was indicated by absence of hematopoietic depression before late death, and by the long duration of tolerance of tissues of the homologous marrow donor antigenic type as revealed by skin grafts. Because of this, and the absence of characteristic late mortality after good early protection with isologous marrow or F_1 hybrid homologous marrow in an irradiated parent strain, it was suggested that the late mortality might more likely be the result of the transplanted homologous hematopoietic tissue reacting immunologically against the foreign tissue antigens of its new host(8). It is well established that the homograft reaction is an immunological reaction against foreign tissue antigens mediated by lymphoid tissue elements, and that an F_1 hybrid of 2 inbred strains of mice will accept tissue transplants from either parent strain, but neither parent strain will accept tissue transplants from the F_1 hybrid. Only the lymphoid system from the same inbred strain or from an F_1 hybrid into a parent strain should not be immunologically active against the tissues of the irradiated recipient. These were the very combinations that failed to give characteristic late mortality after good early protection. Conversely, the lymphoid system of a parent strain transplanted into an F_1 hybrid should react immunologically against the F_1 tissue. The latter system provides an additional test of the theory of late mortality being the result of reaction of marrow graft against the host. While F_1 marrow in the irradiated parent should not result in such late mortality, parent marrow into the irradiated F_1 hybrid should. Such experiments constitute the basis of the present report. In addition, since each parent strain reacts against a different set of tissue antigens in the F_1 hybrid, one group of irradiated F_1 hybrids was given a mixture of half the usual dose of marrow from each of both parent strains, to see if this might give more late mortality than marrow from either strain alone. Also, since tolerance to homologous tissues can be abolished by introducing normal host type lymphocytes(9), an attempt was made to forestall late mortality with foreign-strain homologous marrow by administering also isologous marrow.

Materials and methods. Symbols used for mouse strains, and irradiation conditions were similar to those previously reported(8,11) unless otherwise stated. Mice were randomized by age and sex between treatment groups. Wherever possible an equal number of mice on each of the treatments of a given experiment were housed together in one pen. Skin grafts were performed 174 to 175 days post-irradiation. The LD_{50} for the CBA strain under the conditions of irradiation used is 600 r(11). In all of the experiments herein reported, a single dose of 770 r whole-body X-irradiation was employed, which dose is above the LD_{100}. Bone marrow was injected intravenously in a standard volume of 0.5 ml per mouse. The nucleated cell count of the suspensions used averaged 59,800 cells per cu mm (32,000 to 89,000).

Results. Fig. 1 shows that all of 60 CBA mice receiving 770 r and no marrow were dead by the 12th day. Of the 192 mice similarly irradiated but receiving any of the 4 types of marrow treatment, all groups showed excellent 21-day protection against the lethal effects of irradiation. Beyond 21 days only marrow from a foreign strain (Cb) gave characteristic late mortality. This same marrow mixed with isologous marrow gave no late mortality. Marrow from the F_1 hybrid of the irradiated strain gave protection equal to that with isologous marrow, no characteristic late mortality being observed in either case. Tolerance to grafts of homologous Cb skin was exhibited by all 10 mice so grafted that had been protected with either Cb or (Cb x CBA) F_1 hybrid marrow. Such tolerance was not exhibited by any of the 23 CBA mice so grafted that had been protected by either CBA marrow or a mixture of Cb and CBA







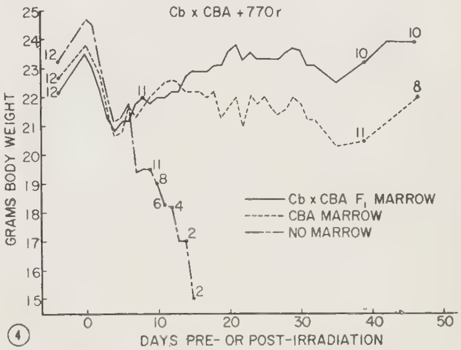


FIG. 1. Cumulative mortality in CBA mice receiving 770 r whole body irradiation followed by isologous, homologous or mixed isologous and homologous bone marrow.

FIG. 2. Cumulative mortality in (Cb × CBA) F_1 hybrid mice receiving 770 r whole body irradiation followed by isologous bone marrow or homologous marrow from either parent strain.

FIG. 3. Cumulative mortality in (A × Db) F_1 hybrid mice receiving 770 r whole body irradiation followed by isologous bone marrow or homologous marrow from either or both parent strains.

FIG. 4. Mean body wt of (Cb × CBA) F_1 hybrid mice receiving 770 r whole body irradiation followed by no bone marrow, isologous marrow, or homologous marrow from one of the parent strains. Each group consisted of 12 mice at the start. Numbers along each curve represent decreasing numbers of surviving mice.

marrow. When either the (Cb x CBA) or (A x Db) F_1 hybrids were protected with isologous marrow, no late mortality after early protection was obtained (Fig. 2, 3). The pre-5 day mortality seen in the (A x Db) mice protected with isologous marrow should not be confused with late mortality, which occurs after the 21st day. When irradiated (Cb x CBA) F_1 hybrids were protected with marrow from either parent strain, no late mortality was obtained with marrow from the Cb strain and slight late mortality with marrow from the CBA strain. However, when irradiated (A x Db) F_1 hybrids were protected with marrow from either or both parent strains, slight late mortality was obtained with marrow from the A strain, moderate late mortality with marrow from the Db strain and severe late mortality with marrow from both the A and Db strains combined. In those groups of F_1 hybrids showing slight to moderate late mortality after protection with parent marrow, many of the mice that escaped late mortality nevertheless showed body weight depression as compared to the controls receiving isologous marrow (Fig. 4). Expressed in terms of potential histo-incompatibility of donor and recipient, late mortality after good early protection against an otherwise lethal dose of irradiation was not seen in those combinations where neither the host nor the donor can potentially react immunologically against each other (CBA into CBA, (Cb x CBA) into (Cb x CBA), (A x Db) into (A x Db)), or where only the host strain can potentially react against the donor strain but not vice versa ((Cb x CBA) into CBA). It was seen in those combinations of foreign strains where both the host and the donor strains can potentially react against each other (Cb into CBA in this report; Db into A, C_3H into CBA, A into CBA, and A into C_{57} in an earlier report)(8). It occurred in some but not all of those combinations where only the donor strain can potentially react against the recipient strain but not vice versa (Cb into (Cb x CBA); CBA into (Cb x CBA); A into (A x Db); Db into (A x Db) and both A and Db into (A x Db)). The term "potentially" is used advisedly since a host potentially capable of reacting against and rejecting the tissues of a homologous strain is rendered incapable of doing so by protection against an otherwise lethal dose of irradiation with marrow of that homologous strain, as demonstrated by the survival of skin and bone marrow. There is no evidence, however, that transplanted homologous lymphoid elements are thereby rendered incapable of reacting against the tissues of the host.

Discussion. The absence of characteristic late mortality after good early protection in irradiated CBA mice given homologous marrow from an F_1 hybrid of the CBA strain, and its presence in CBA mice protected with homologous marrow from a foreign strain (Cb), indicates that late mortality is not the result of recovery of the host's ability to react immunologically against the grafted homologous marrow elements. The CBA strain normally reacts against and rejects tissue grafts from either the Cb strain or the (Cb x CBA) F_1 hybrid, since both contain the same set of foreign antigens characteristic of the Cb strain. Yet late mortality is seen only with Cb marrow. This, together with the absence of secondary hematopoietic depression before late death, and the induced tolerance to tissues of marrow donor antigenic type as revealed by skin grafts, contradicts the concept that late mortality results from the host reacting against the marrow graft, at least under the conditions of the present experiments. In this regard it must be emphasized that the level of irradiation employed was above the LD_{100}. It is highly probable that at lower levels of irradiation some of the irradiated host's cells responsible for the homograft reaction would recover and reject homologous tissue, whether marrow or skin(11). There is one difference between the Cb and the (Cb x CBA) F_1 marrow donor that correlates well with the observed result. Lymphoid elements of the Cb strain can react against the tissues of the CBA recipient, whereas those of the (Cb x CBA) F_1 hybrid cannot.

The absence of late mortality in CBA mice protected with a mixture of Cb and CBA marrow dissociates late mortality from any immediate effect of the homologous marrow on the host, including transmission of pathogenic bacteria. Absence of late mortality in this group would appear to be related to failure of the Cb marrow to survive when administered with isologous marrow. That it could not survive in this group is indicated by rejection of Cb skin grafts in all 11 mice of this group so tested. As indicated earlier, tolerance of homologous marrow donor tissues can be abolished by subsequent administration of isologous (host type) lymphocytes(9). It would appear that tolerance can be prevented initially by simultaneous administration of homologous and isologous marrow. Implicit in these results is the concept that late mortality is related to the continued persistence and function in the irradiated host of marrow elements from a homologous donor that is capable of reacting immunologically against the recipient. The presence of characteristic late mortality in F_1 hybrids protected with marrow from some but not all parent strains is difficult to interpret. Its absence cannot be associated with the use of 2 parental strains of the same H-2 genotype, since the parent strains of both hybrids differed at the H-2 locus(12). That characteristic late mortality occurs in any group of F_1 hybrids protected with parent strain marrow is still further evidence that late mortality does not result from recovery of the host's ability to react against the grafted marrow elements, for the F_1 hybrid could not so react to begin with. But if late mortality represents an immunological reaction of the graft against the host, then it should occur in all the combinations of parent marrow into F_1 hybrid. Unless, of course, the hypothesized reaction of transplanted hematopoietic elements against the tissues of a tolerant host does not always result in death. It is possible that if the degree of incompatibility is small enough, and the tolerant host strong enough (heterosis), the great excess of antigen and constant replacement of damaged tissue may permit the host to survive in spite of an incompatibility which when operating in the direction of host *vs.* donor would uniformly cause rejection of skin grafts. In human blood transfusion, a given blood type incompatibility which when operating in the direction of patient *vs.* donor usually gives a major transfusion reaction, will, when operating in the direction of donor *vs.* patient usually give only a minor transfusion reaction. It would seem appropriate to look toward signs in the process leading to late mortality, before the severe end-point of death itself. The fact that in some combinations of homologous donor and recipient strains not all of the treated mice show late mortality but the survivors may show a body weight depression, suggests that the "disease" in question is not always a lethal one. Impaired hair growth, atrophy of lymphoid organs and general debilitation as well as body weight depression are characteristic of irradiated mice treated with foreign strain homologous or heterologous marrow as opposed to isologous marrow, even though they may not succumb to characteristic late mortality during the 60-day post-irradiation period.

If late mortality represents the reaction of transplanted homologous or heterologous hematopoietic tissue against a tolerant host, then irradiation is not an essential component of the disease, except insofar as it is necessary to render the host tolerant. If tolerance could be achieved by other means, such a disease should be demonstrable even in unirradiated animals. This would be an important step in establishment of the etiology of the disease. Actually immunological tolerance can be achieved by introduction of homologous or heterologous tissues into unirradiated animals of several species before or shortly after birth or hatching(13). In addition, F_1 hybrid mice are normally tolerant of tissue grafts from either parent strain. Experiments on the latter system are now in progress. In the former system, Billingham and Brent(14) have observed delayed mortality. The following points of similarity between the condition they observed and that seen after irradiation protection with homologous marrow strongly suggest that the two represent the same process: a) appearance of the condition following introduction of homologous lymphoid elements or their precursors into tolerant hosts; b) delayed onset of overt manifestations; c) depression of body weight; d) lymphoid organ atrophy, and e) greater occurrence of mortality in some combinations of donor and recipient strains than in others. The possible identity of these 2 conditions, together with the absence of characteristic late mortality in irradiated mice protected with isologous or F_1 hybrid homologous marrow would prompt one to avoid use of the terms "late *irradiation* mortality" or "secondary *irradiation* syndrome." It would appear more appropriate to use the terms "homologous disease" and "heterologous disease" to designate the specific complications attributable to successful transplantation of homologous or heterologous, as opposed to isologous or autologous bone marrow or lymphoid tissue into a tolerant host. This would apply to those situations where tolerance is induced or acquired, regardless of the method, as well as where tolerance is a congenital abnormality or a genetically determined normal state. In addition to the conditions already observed in irradiated or neonatally injected animals it would include any similar conditions subsequently observed in a variety of situations, such as agammaglobulinemic or hypogammaglobulinemic individuals transplanted with lymph nodes, as well as in F_1 hybrid mice receiving bone marrow or spleen or lymph nodes from either parent strain.

Summary. CBA mice protected against an otherwise lethal dose of irradiation with isologous CBA bone marrow showed normal rejection of skin grafts from the Cb strain. Irradiated CBA mice protected with Cb or (Cb x CBA) F_1 hybrid marrow were tolerant of skin grafts from the Cb strain. Irradiated CBA mice protected with a mixture of marrow from the Cb and CBA strain were not tol-

erant of skin grafts from the Cb strain. Late mortality (21 to 60 days post-irradiation), after good early protection (21 days) against doses of whole body X-irradiation above the LD_{100}, did not occur in mice protected with isologous bone marrow (where neither the host nor the donor can react immunologically against each other). It occurred in mice protected with homologous marrow from a foreign strain (where both the host strain and the donor strain can potentially react immunologically against each other). It did not occur in mice protected with marrow from an F_1 hybrid of the irradiated strain (where the host strain can potentially react against the donor strain but the donor strain has no potential to react against the host strain). It occurred in some but not all combinations of irradiated F_1 hybrids protected with marrow from a parent strain (where the host has no potential to react against the donor strain but the donor strain can potentially react against the recipient strain).

The terms "homologous disease" and "heterologous disease" are proposed to designate the complications specifically attributable to successful transplantation of homologous or heterologous, as opposed to isologous or autologous, bone marrow or lymphoid tissue into a tolerant host.

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Production of Hyaluronic Acid in Tissue Culture by Fibroblasts Growing from Intestinal, Stomach, Liver and Kidney Explants.* (23415)

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The isolation of hyaluronic acid, and the description of its enzymatic hydrolysis by Meyer(1) brought to light the enzyme-substrate nature of the spreading reaction, first found in skin and subcutaneous tissue(2). In many tissues in which the spreading reaction was demonstrated, hyaluronic acid was found to be present and to be hydrolyzed by hyaluronidase. On the other hand, spreading factors were described which were not found to be related to hyaluronic acid and its hydrolysis, so-called hyaluronate-inactive spreading factors.

Favilli(3) and Duran-Reynals(2) demonstrated the spreading reaction in organs other than skin and subcutaneous tissue, such as stomach, intestine, and striated muscle, while no conclusion could be reached concerning spreading reaction in hepatic and renal tissue. It was, however, unknown whether the spreading reaction found in those organs was

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* Aided in part by U.S.P.H.S. grant.

TABLE I. Positive Mucin Clot Test in Supernates of Cultures of Fibroblasts Growing from Intestinal, Stomach, Liver and Kidney Explants.

			Tubes after 10 days		Tubes after 30 days		After 38-96 days	
Organ	Growth	Tubes	Good growth	Mc +	Good growth	Mc +	Good growth	Mc +
Liver	Mostly fibroblasts; some liver epithel.	34	28	14	26	24	16	14
Stomach	Fibroblasts and epithel. membranes	30	22	18	24	18	9	8
Intestine	Epithel. and fibroblasts	12	10	7	11	8	4	4
Kidney	Fibroblasts; tubuli and epithelial sheets	10	10	6	10	8	7	5

hyaluronate-active and, if so, whether or not hyaluronic acid present in those organs was produced locally. These questions could most adequately be studied in tissue culture experiments.

Methods. Tissue cultures were prepared from liver, stomach, intestine, and kidney of 10- to 12-day-old chick embryos on a thin layer of chicken plasma, to which after coagulation, a liquid supernate was added consisting of 20% chick embryo extract, 30% horse serum, 20% chick amniotic fluid, and 30% Earle's balanced salt solution. The medium was renewed every 3 to 4 days in the first 3 weeks of cultivation, later every 6 days. For testing the presence of hyaluronic acid in the culture medium, the method of mucin clot (mc) formation and its prevention by testicular hyaluronidase was used. Formation of a tight mc on addition of 0.2 ml of 1 N acetic acid to 1 cc of the culture medium indicated the presence of hyaluronic acid, if formation of the mc could be prevented by hyaluronidase. Since chondroitin sulfates and partially depolymerized hyaluronic acid do not yield a mc on addition of acetic acid, this test has proven convenient for obtaining prompt information concerning the presence of highly polymerized hyaluronic acid in small samples of tissue culture supernates(4).

The minimal concentration of hyaluronic acid which gives a positive mc test is about 30 μg/ml. Fresh undiluted EE_{50} prepared in this laboratory gave a mc which could be prevented by hyaluronidase. EE_{50} contained about 105-120 μg hyaluronic acid per ml; 20% of it contained about 20 μg/ml, which is below the minimal concentration for the mc test and in fact never gave a mc. On the other hand, it has been found that hyaluronic acid, contained in chick EE in any concentration, is depolymerized at 37° in the first few days of incubation (Table II). Consequently, a positive mc test after that time always indicates the presence of hyaluronic acid newly produced *in vitro* by fibroblasts. Chick EE may thus be used in any concentration in the culture medium in which production of hyaluronic acid *in vitro* will be tested by the mc method.

Results. Growth of cultures from chick embryo stomach and intestine was luxuriant and large epithelial membranes predominated. Each culture, however, showed also more or less growth of fibroblasts. Explants from chick embryo liver gave abundant growth predominantly of fibroblasts, and about 40% of the cultures showed, in addition, growth of typical liver epithelium, which, however, degenerated in 1 to 2 weeks while fibroblasts continued to grow.

The explants from kidney of 10- to 12-day-old chick embryos were either mesonephric or metanephric, or both. Microscopical observation of cultures showed profuse growth of epithelial sheets typical in cultures of embryonic kidney, with elongated cells (kidney epithelium is of mesodermal origin), but rather small nuclei. In most cultures, growth of mostly strait tubuli were observed, and in some cultures of metanephros scattered epithelial islets. In addition, in most cultures scarcely growing typical fibroblasts were observed.

Starting from the 3rd or 4th, sometimes only after the 7th renewal of the medium, the supernate of those of the cultures which showed good growth, on addition of a few drops of 1 N acetic acid, yielded a tight mc, the formation of which could be prevented by hyaluronidase. The feeding solution gave no mc before incubation (Table I). Estimation of hyaluronic acid produced in cultures of the named tissues, using the turbidimetric method, gave values ranging from about 36 to 210 μg per ml in various cultures, and at different times and intervals between feedings. The production rate was evidently dependent on the state of the cultures. Factors influencing production were not further analyzed in these experiments.

Discussion. In previous reports(4,5), production of hyaluronic acid in tissue culture by fibroblasts growing from explants of human synovia, human embryo skin, bone, tendon, epiphysis, aorta, and pericardium; rat subcutaneum; chick embryo bone and heart, and beef embryo bone was demonstrated. The present results show that hyaluronic acid is produced in tissue culture of liver, stomach, intestine, and kidney of chick embryos by cells of mesenchymal origin, since all cultures, the supernate of which gave an mc, showed either growth of fibroblasts alone, or growth of fibroblasts mixed with epithelial growth, while the supernate from cultures with pure epithelial growth never gave an mc. The present findings also indicate the local production of hyaluronic acid in chick embryo liver, stomach, intestine and kidney.

It should be emphasized that, for studies of hyaluronic acid production *in vitro,* the use of primary cultures is the method of choice. Such cultures have been producing hyaluronic acid for nearly one year and under suitable conditions may perhaps produce indefinitely. If production stops (for unknown reasons), it may soon start again, under more suitable conditions of growth. On the other hand, replica cultures of cell strains of mesenchymal origin sooner or later irreversibly lose their capacity of hyaluronic acid production *in vitro,* as is the case, for instance, with strain L, deriving from a mouse fibroblast, and with similar strains of mesenchymal origin. It appears that such strains (as a result of trypsinization or other procedures leading to a loosening of cell connections) gradually lose their ability to produce mucopolysaccharides as precursors of ground substance, or in other words, gradually lose their capacities to form connective tissue.

It has yet not been possible to identify factors which in tissue culture specifically promote hyaluronic acid production. In general, conditions which are required for obtaining good growth are also necessary for continuous production of hyaluronic acid. Deterioration of cultures due, for instance, to pH changes or to starvation will impair both growth and production of hyaluronic acid. Similarly hydrocortisone in high concentrations which inhibit growth promptly inhibit also production of hyaluronic acid. On the other hand, it appears that there are specific factors which promote hyaluronic acid production only but not growth. In preliminary work cultures of fibroblasts were kept for several weeks at room temperature. In such stationary cultures, despite significant or complete depression of growth, production of hyaluronic acid continued in some cultures. By cultivation at lower temperatures, factors promoting hyaluronic acid production as distinct from growth factors, could thus more conveniently be studied.

Summary. 1. Tissue cultures of chick embryo liver, stomach, intestine and kidney, in which both epithelium and fibroblasts were growing for over 3 months produced hyaluronic acid, as shown by a positive mucin clot test and by the prevention of mucin clot formation by hyaluronidase. These findings are consistent with the demonstration of the spreading reaction in some of these organs and indicate that they belong to the group of hyaluronate-active spreading reactions. 2. In primary cultures production of hyaluronic acid may take place for very long periods of

TABLE II. Incubation of Chick Embryo Extract (EE_{50}) at 37°C.

No. of samples	Before incubation	Days of incubation	No mucin clot
7	Mucin clot +	1	4
14	"	2	12
11	"	3	9
12	"	4	11
25	"	5	22

TABLE IIa. Incubation of Supernates from Tissue Cultures.

Tissue	Medium tested before feeding	After 5 days of growth	Days of incubation of supernates alone at 37°C	# samples	Mc +	No mc
Rat subcut.	No Mc	Mc +	4 - 18	9	9	—
Chick embryo bone	”	”	8 - 33	41	38	3
Rat tail tendon	”	”	4 - 55	7	6	1
Rat subcut.	”	”	30*-195*	3	3	—
Chick embryo bone	”	”	6*- 65*	22	21	1

* Kept in refrigerator at 4°C.

time, usually as long as the cultures do not deteriorate, while replica cultures, for instance strain L, sooner or later lose the ability to produce hyaluronic acid, despite their excellent appearance and good growth. 3. Although it has not yet been possible to separate factors promoting mucopolysaccharide production from factors promoting growth, it has been observed that cultures kept at room temperatures with considerably reduced growth, may sometimes continue to produce hyaluronic acid. 4. Hyaluronic acid of chick embryo extract is depolymerized in the first few days of incubation at 38°C (Table II), while hyaluronic acid produced in tissue culture is not depolymerized for months under the same conditions (Table IIa). A positive mucin clot test after a few days of incubation thus always indicates the presence of hyaluronic acid newly produced *in vitro* by fibroblasts. Chick embryo extract may therefore be used in any concentration in the culture medium in which production of hyaluronic acid *in vitro* will be tested by the mucin clot method.

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Calcification XIX. Calcification of Transplanted Rachitic Bone.*† (23416)

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The purpose of this investigation was to study calcification of rachitic bone slices that were transplanted subcutaneously to rats with the thought that transplantation would be a useful approach to the study of the calcifying mechanism as was the case with *in vitro* calcification(1-10). By standardizing both the rachitic sections and the host animal, a transplantation technic would permit the study of calcification after experimental modification of either the rachitic section or the composition of body fluid in the host animal.

Experimental. Method of preparation of animals for the transplants. Each animal was anesthetized with ether and placed on its back. The skin covering the abdomen was cleansed with 70% alcohol and a 12 to 15 mm incision was made along the medial ventral line about 20 mm anterior to the genital organ. All instruments and sutures were sterilized prior to use and sterile precautions were adhered to in the handling of the transplants. After the incision was made, the transplant was placed into a subcutaneous pocket by lifting the skin and pushing the transplant with a blunt end forceps laterally from the line of incision to a distance of about 20 mm. The incision was closed with 3 or 4 stitches. Each animal was housed in a separate cage for the duration of the experiment. Antibiotics were not used and in only one instance was infection encountered.

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Two groups of experiments were carried out. An inbred strain of Wistar rats was used.

Group one consisted of transplanting rachitic tibial slices into rats that were 6 to 8 weeks of age. Half of the rats were rachitic and half were normal. The rachitogenic diet was composed of 75% degerminated corn meal, 19% wheat gluten, 2% Brewer's yeast, 3% calcium carbonate and 1% sodium chloride. The normal diet was that of Bills *et al.* (11).

The tibial slices used for transplantation and for *in vitro* calcification were obtained from rats that had been maintained on the aforementioned rachitogenic diet for 3 to 4 weeks after weaning and were prepared, under sterile precautions, as previously reported (12). Three, frontal plane and longitudinally sliced sections were obtained from each tibia. One section was used to test for rickets using the silver line test as described in the next paragraph. The second section was transplanted to a rat that had been fed the rachitogenic diet for 3 weeks after weaning and the third section was transplanted to a rat that had been fed the normal diet for 3 to 4 weeks after weaning. Separate transplants were made to at least 5 individual animals on each of the diets.

The following procedure was used to test for rickets. A section was placed in a concavity of a glass spot plate, covered with 3% silver nitrate solution and exposed to light from a 100 watt tungsten filament lamp for 5 minutes. The distance from the section to the lamp was about 8 inches. After exposure, the section was examined with a Leitz wide field stereobinocular microscope at a magnification of 32 X. If healing was observed, as evidenced by a black silver stain in the rachitic metaphysis, the sections from that animal were discarded.

A similar procedure (line test) was used to determine degree of calcification of each of the experimental sections in this study. The evaluation and the procedural details have been described by Sobel *et al.*(13,14).

The slices from the other tibia were subjected to *in vitro* calcification for 18 hours as described previously(9,14). The *in vitro* calcifying solution was prepared by adding to a one liter volumetric flask, 100 ml of a stock basal salt solution‡ and 50 ml of a NaH_2PO_4 - Na_2HPO_4 solution that had a phosphorus concentration of 1 mg/ml. Five hundred ml of glass distilled water was added and the pH lowered to about 6.3 by aerating with 100% CO_2. To this solution were added either 2 ml or 5 ml of specially prepared calcium chloride solution that had a calcium concentration of 20 mg/ml. Thus, the calcifying media had Ca x P products of 20 and 50, *i.e.*, 4 mg% Ca x 5 mg% P, or 10 mg% Ca x 5 mg% P. Distilled water was added to the liter mark. The pH of the final *in vitro* calcifying solution was adjusted to 7.3 by aerating with 100% N_2.

In addition to the *in vitro* calcification in purely inorganic media, *in vitro* calcification of tibial slices was performed in sera obtained from rachitic and from normal rats. Blood from partially anesthetized animals was obtained from the severed jugular vein and collected in test tubes that contained 3 ml of sterile mineral oil. The tubes were centrifuged and the sera aspirated, pooled and thoroughly mixed. The pooled sera, maintained under oil at all times had a pH of 7.32. The calcium content was determined by the method of Sobel and Sobel(15) and the phosphorus by the Fiske and Subbarow method (16). The pooled sera from the rachitic rats and the pooled sera from the normal rats were aseptically transferred in four 10 ml portions for each of the sera to 12.5 ml capacity plastic capped vials. Mineral oil was added to fill each vial and the cap was kept in place by the use of rubber bands stretched tightly over the cap and under the bottom of the vial. After incubation at 37°C for 18 hours, the cap was removed and the mineral oil aspirated off. The tibial slice was washed with distilled water and subjected to the silver line test.

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‡ The stock basal salt solution contained 0.7 M NaCl, 0.5 M KCl and 0.22 M $NaHCO_3$/l. It was Berkefeld filtered and stored in a pyrex bottle.

In one of the experiments, pooled rachitic serum was divided into 2 portions. One portion, unmodified, had a Ca x P product of 15.9. The other portion was modified by increasing the phosphorus content from 1.5 mg% to 5.0 mg% by the addition of inorganic phosphate. Thus, the Ca x P product of this serum was increased from 15.9 to 53.0. A rachitic slice was suspended in each of 2 vials containing 10 ml of the unmodified serum and in like manner, a rachitic slice was suspended in each of 2 vials containing 10 ml of the phosphorus enriched serum. A layer of mineral oil was placed over the serum and each vial was capped and incubated as mentioned above. The degree of *in vitro* calcification (as the average of 2 slices for each serum) is shown in Table II.

In the second group of experiments, 20 slices of rachitic tibiae were divided into 2 portions. One portion, consisting of 10 slices placed separately into 10 sterile test tubes of 15 ml capacity and stoppered with sterile rubber stoppers was shaken mechanically for one hour in 150 meq/l $CaCl_2$ at 24-26°C. The calcium chloride solution was Berkefeld filtered just prior to use. After shaking, the slices were washed with freshly distilled water to remove the excess of calcium chloride. The second portion was not treated with calcium chloride. Both portions were transferred to sterile vials and stored for 2 weeks at –22°C ± 2° in a deep freeze chest. After the 2 week period of storage, one calcium treated and one untreated slice was implanted under the skin of each animal laterally and on opposite sides of the medial ventral line incision. The animals were 7 to 8 weeks of age and had been maintained on the normal diet. After 1, 2 or 3 weeks following the transplantations, the animals were sacrificed by chloroform asphyxiation. The transplants were removed and examined for degree of calcification in the rachitic metaphysis. The *in vitro* calcification of similarly prepared deep freeze stored tibial slices was carried out as described previously(17). The *in vitro* calcifying solution had a Ca x P product of 50 (10 mg% Ca x 5 mg% P).

Results. There was no evidence of calcification of the bone transplants that were placed into the rats on the rachitogenic diet. In contrast, the transplants in the rats on the normal diet showed a progressive increase in calcification from week to week and a simultaneous decrease in the width of the uncalcified epiphyseal cartilage. After one week the calcification in the provisional zone of the hypertrophic cartilage was 1.3(+++). After 2 weeks the calcification was 2.5(++++) and after 3 weeks 3.5(++++). All of the transplants in the animals on the normal diet were encapsulated with fibrous tissue. Removal of the capsule revealed an extremely hard transplant which could not be readily cut with a scalpel. This was especially true for the transplants that were placed into the animals for 2 and 3 weeks. In all instances the metaphyseal osteoid was well calcified too. On several occasions, ridges similar in hardness to compact bone developed along the edges of the slices. This material produced a dense, black silver stain when immersed in silver nitrate solution and exposed to light. The transplants excised from the rats on the rachitogenic diet were less encapsulated and removal of the capsule revealed a soft transplant which could be easily cut with a scalpel.

Here we have an example of one way in which transplantation can be used in calcification studies. The difference in calcification of the same type of rachitic bone is due to the differences in the body fluid brought about by different diets. In these studies it was possible to establish that the predominant factor that altered calcification was the Ca x P product of the serum, 16.7 for the rachitic animals and 89.8 for the normal animals (Table I).

TABLE I. Calcification of Preosseous Cartilage of Rachitic Tibial Slices Transplanted to Rats on Rachitogenic and Normal Diets.

Diet	Blood serum Ca, mg %	 P, mg %	Ca × P product	Degree of calcification after 1 wk	 2 wk	 3 wk
	Mean					
Rachitogenic	9.8	1.7	16.7	0 (0)	0 (0)	0 (0)
Normal	10.2	8.8	89.8	1.3(+++)*	2.5(++++)*	3.5(++++)*

* Calcification was observed in the metaphyseal osteoid and was most extensive after 3 wk.

Table II summarizes the *in vitro* calcification results. When the serum of rachitic animals was reinforced by added phosphate, thus raising the Ca x P product, calcification *in vitro* occurred just as it did in the serum of normal animals. With unmodified rachitic serum *in vitro* calcification did not occur. This is parallel to the findings of *in vitro* calcification in inorganic media where calcification took place at the higher product.

TABLE II. Calcification of Preosseous Cartilage of Rachitic Tibial Slices *In Vitro* for 18 Hours in Inorganic Medium and in Serum.

Ca × P product	Ca, mg %	P, mg %	Degree of calcification
Mean			
Inorganic medium			
20	4.0	5.0	0 (0)
50	10.0	5.0	1.5(++++)
Rachitic rat serum			
15.9	10.6	1.5	0 (0)
53.0	10.6	5.0*	1.1 (+++)

* The phosphorus in this serum was raised from 1.5 mg % to 5.0 mg % by the addition of inorganic phosphate.

In this manner, factors other than the serum Ca x P product favoring or retarding calcification may be detected in the future. For example, animals under the influence of drugs, hormones or hereditary differences may contain these factors.

In the second group of experiments in which deep freeze stored bone slices, with and without prior treatment with calcium chloride were placed into animals on a normal diet, the treated slices showed a more rapid rate of calcification in the rachitic metaphysis. The results are shown in Table III. The treated transplants excised after one week in the animals showed 1.4(++++) calcification in the epiphyseal cartilage while the untreated transplants showed only a trace. After 2 weeks, the calcium treated transplants were not only more densely calcified than the untreated transplants but marked calcification was observed in the metaphyseal osteoid in addition to that in the epiphyseal cartilage. After 3 weeks, the untreated transplants still showed a wider uncalcified epiphyseal cartilage as compared to the calcium treated transplants. The latter showed a 3. (++++) calcification in the epiphyseal cartilage and extensive calcification in the metaphyseal osteoid while the untreated transplants showed a 1.5(++++) calcification in the epiphyseal cartilage and only traces in the metaphyseal osteoid.

Since the animals in this experiment were fed the same normal diet and since each animal received one calcium treated and one untreated transplant the difference in degree of calcification of the transplants may be as-

TABLE III. Calcification of Preosseous Cartilage of Transplanted Rachitic Tibial Slices Treated and Untreated with Calcium Chloride Prior to Deep Freeze Storage at −22°C ± 2° for 2 Weeks.

Treatment of slices prior to deep freeze storage	Degree of calcification after: 1 wk	 2 wk	 3 wk
	Mean		
None	.7 (+)	.9 (+++)	1.5 (++++)
Shaken 1 hr at 24-26°C in 150 meq/l of $CaCl_2$	1.4 (++++)	2.3 (++++)*	3.0 (++++)*

* Extensive calcification was observed in the metaphyseal osteoid.

TABLE IV. *In Vitro* Calcification of Preosseous Cartilage of Rachitic Tibial Slices Treated and Untreated with Calcium Chloride Prior to Deep Freeze Storage for 2 Weeks.

Treatment of slices prior to deep freeze storage	Degree of calcification after 18 hr ($Ca \times P = 50$)
	Mean
None	0 (0)
Shaken 1 hr in 150 meq/l $CaCl_2$	1.5 (++++)
Fresh, untreated and not deep frozen	2.0 (++++)
Fresh, unfrozen, shaken 1 hr in 150 meq/l $CaCl_2$	2.5 (++++)

cribed to the differences in the bone rather than the body fluid. The *in vitro* calcification studies in pure inorganic solution showed that the deep freeze (not calcium treated) stored slices would not calcify at a Ca x P product of 50 in solutions containing 5 mg% P either as inorganic or organic phosphorus while the calcium treated slices calcified at this product (Table IV). It is apparent from these findings that the calcifying mechanism is inactivated in the untreated deep freeze stored bones and this inactivation can be prevented or reversed with Ca ions(17). The transplants showed a partial restoration of the calcifying mechanism in the deep freeze stored bone slices which may indicate that possibly the body fluids contain some ingredient which, if present in the *in vitro* fluid, might have been manifested in calcifiability.

These studies with deep freeze stored bones exemplify the influence on the calcifying mechanism of bone treated in various ways and which may be tested under conditions that exist in a standard body fluid, such as that of a normal animal. This method is not limited only to rachitic bone taken out and treated thereafter but could conceivably use excised rachitic bone produced in different ways such as in Sr rickets(5), Be rickets(4) or under the influence of hormones, such as parathormone or rickets produced in animals by thyroid-parathyroidectomy(18). Testing such bones only by *in vitro* calcification may miss significant information that may be discovered by transplantation.

The usefulness of transplantation is indicated in some of our other studies with demineralized bone, with and without chondroitin sulfate treatment, and treated in various ways(19) which showed that calcification will occur where *in vitro* calcification does not appear to occur (to be published elsewhere). Another study showed that ATP plus Ca^{++} treated bones calcified *in vitro* at lower products(20,21) and mineralized when transplanted to a rachitic animal in which the animal's own bones would not mineralize.

Summary. Calcification of the rachitic metaphysis, with typical silver line test, was obtained in tibial slices that were transplanted to rats on a normal diet. Similar transplants to rats on a rachitogenic diet did not calcify. This difference was probably due to the higher Ca x P product in the body fluids of animals on a normal diet as compared to the product of animals on a rachitogenic diet. *In vitro* calcification was obtained in the sera of normal animals, in the sera of rachitic animals to which phosphate was added to raise the Ca x P product but not in the unmodified sera of rachitic animals. *In vitro* calcification was obtained in inorganic solutions with a Ca x P product of 50 but not with a Ca x P product of 20. Transplanted deep freeze stored bone slices treated with calcium chloride prior to deep freeze calcified more intensively than deep freeze stored bones not treated with calcium, as measured by the silver line test. Calcification *in vitro* studies indicated reversible inactivation of calcifiability of stored frozen bone which could be prevented or restored by calcium chloride treatment. While differences existed, calcification of the deep freeze stored bone indicated that there was a partial restoration of the calcifying mechanism or that some unknown ingredient exists in the body fluids which, if present, could have elicited restoration of the calcifying mechanism. Transplantation of rachitic bone, as proposed, may be an additional method for studying the differences of the actual body fluids on calcification of a given rachitic bone, or after treatment of such bone the changes in calcifiability with an actual standard body fluid such as that of an animal on a normal diet.

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Antihemophilic Factor (AHF): Plasma Levels After Administration of AHF Preparations to Hemophilic Dogs.* (23417)

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Intravenous administration of plasma fractions rich in antihemophilic factor (AHF) has been shown to be followed by a rapid rise in the plasma level of AHF. In normal and hemophilic dogs, half of the plasma AHF was lost in 2-6 hours(1,2). A similar rise and fall in AHF was observed in human hemophiliacs following injections of large volumes of plasma(2) or bovine AHF fractions(3). Recently it was shown that high plasma AHF levels occurred following intravenous injection of a human plasma fraction(4). Similar quantitative studies on other routes of administration of AHF are not available, although many years ago it was observed(5,6) that the clotting time in hemophilia was shortened after intramuscular injections of a plasma globulin substance. Other workers (7,8) indicated subsequently that intramuscular injections were both ineffective and dangerous. Data presented in this paper demonstrate the relative effectiveness of various routes of administration of a plasma AHF fraction to hemophilic dogs. After each injection, plasma tides of AHF were determined. A preliminary report of some of these data was given earlier(9).

Materials and methods. Lyophilized bovine AHF was prepared by a modification of Bidwell's procedure(10). On the basis of protein and AHF determinations the prepara-

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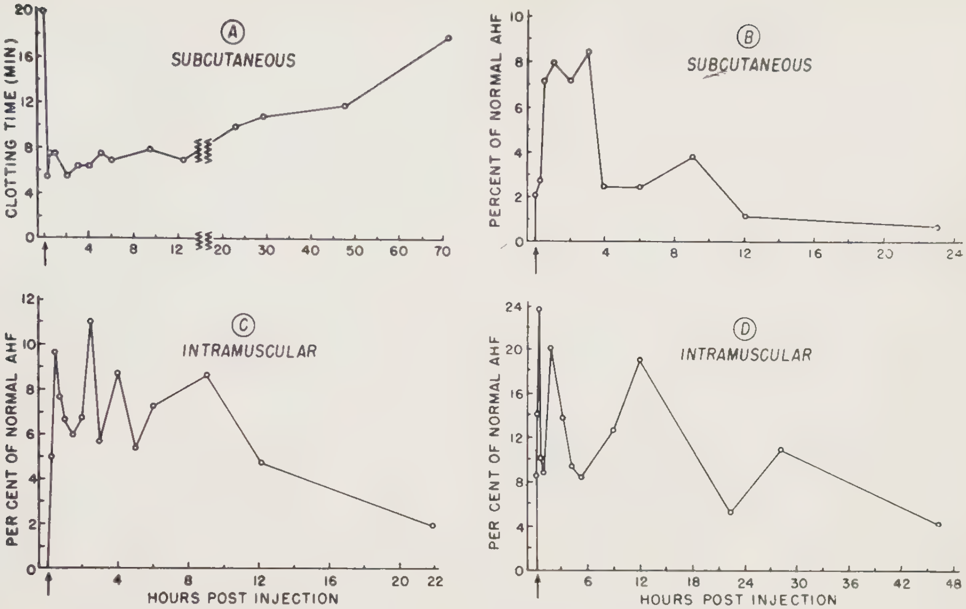

FIG. 1. AHF levels following injections of AHF preparations. *(A) Subcut.:* 3.5 kg female hemophilic dog was given 19 ml AHF preparation dissolved in citrate-saline solution, 27 times the activity of dog plasma, in 4 equal doses, each thigh. Inj. time 10 min. *(B) Subcut.:* 11.9 kg female hemophilic dog was given 25 ml AHF preparation dissolved in 0.11 M citrate, 19 times the activity of dog plasma, in 4 equal doses, each thigh. Inj. time 4 min. Pre-inj. clotting time 38 min. *(C) Intramusc.:* 3.5 kg female hemophilic dog given 19 ml AHF preparation dissolved in citrate-saline solution, 25 times the activity of dog plasma, in 4 equal doses, each thigh. Inj. time 12 min. Pre-inj. clotting time 26 min. *(D) Intramusc.:* 13.2 kg female hemophilic dog given 62 ml AHF preparation, dissolved in 0.11 M citrate, 15 times the activity of dog plasma, intramusc. Inj. time 11 min.

tions were found to be about 50 times as concentrated as the initial plasma. The dry powder was reconstituted either in *citrate-saline* solution (0.02 M sodium citrate, 0.127 M NaCl) or in *sodium citrate* (0.11 M). AHF activity of the solutions used for injection was 4-27 times greater than normal canine plasma. AHF was injected into hemophilic dogs(11) by subcutaneous, intramuscular, or intravenous routes, and the plasma AHF was assayed periodically(12). Pooled plasma from 3 normal dogs served as the control (100% AHF) in the assays. Whole blood clotting times were done by a modified Lee-White procedure. Normal values were 5-7 min.

Results. Subcutaneous injection. Results of 2 experiments are given. In the first, AHF was injected as a solution in citrate-saline, in the second, as a solution in strong citrate. The details of the first experiment are indicated in Fig. 1A. The dose injected was calculated to be sufficient to raise the plasma AHF to 200% of the normal dog level had it been given intravenously. Actually, the AHF did not reach assayable levels (about 1-3%) at any time during the experiment. Sufficient circulating AHF was present, however, to maintain clotting time at a normal level for many hours. The injection sites became firm but not discolored within an hour after the injections and remained so for 24 hours. In the second experiment (Fig. 1B), the dose, if given intravenously, should have raised the plasma AHF to 80%. Five min after injection AHF was detected in the plasma. The values rose to 7-8% and remained there for 2½ hours. Clotting times, not shown on the figure, were reduced to the normal range for 29 hours. Injection sites remained pliable.

Intramuscular injection. Results of 2 experiments, similar in design to the subcutaneous injections presented above, are shown in Fig. 1. In the first experiment (Fig. 1C),

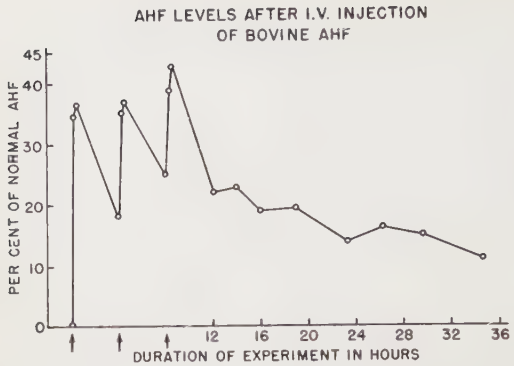

FIG. 2. AHF levels following repeated, closely-spaced intrav. injections of AHF fractions. 12.6 kg female hemophilic dog was given a priming dose of 58 ml AHF in citrate solution, 4 times as active as dog plasma at 0 time. 29 ml doses inj. at 4 and 8 hr respectively. Pre-inj. clotting time 31 min.

the AHF was injected as a solution in citrate-saline; the same dose, if injected intravenously, should have raised the AHF to 200%. Promptly after intramuscular injection, AHF appeared in the plasma; the highest level, 11%, was reached 2½ hours after injection. Clotting times were reduced and remained within the normal range for 2 days. The injection sites became firm. In the second experiment of this group (Fig. 1D), AHF was injected as a solution in strong citrate; the dose if injected intravenously should have raised the AHF to 80%. As before, the AHF appeared promptly in the plasma, but it reached higher levels than in any of the previous experiments. Measurable amounts were still present 46 hours after injection. Clotting times remained normal for the same period. The injection sites in this experiment remained pliable.

Closely-spaced intravenous injections. A series of experiments was performed in which the fall-off in plasma AHF was followed after moderate levels had been maintained for several hours. The results of one experiment are given in Fig. 2. There was good correlation between expected and observed plasma AHF values after each injection. The doses of AHF administered were expected to raise the plasma levels to 37, 36, and 44% respectively; observed peak levels after each injection were 37, 37, and 43%. It will be noted that plasma AHF was maintained at or above the 18% level for 12 hours. After each injection, the decline in AHF was rapid with the level dropping to about one-half the peak value in 4 hours. In contrast to the rapid fall observed in the first part of the experiment, the decline was relatively slow in the last part. Approximately 23 hours elapsed while the AHF fell from 22% to 11%.

Discussion. In comparing the relative efficacy of subcutaneous and intramuscular injection of AHF, it was observed that in either case the factor appeared promptly in the plasma. However, plasma AHF was maintained at higher levels and for longer times after intramuscular injection. It appears likely that the firmness of the injection sites, noted when weak citrate solutions of AHF were used, was due to local clotting of fibrinogen in the bovine AHF fractions. Impaired absorption as well as local destruction of AHF by thrombin(13) may well have occurred in these experiments. Better results were obtained when strong citrate solutions of AHF were used, despite the fact that the doses employed were only 40% as large as those in weak citrate. It has been suggested(1) that in hemophilia "an AHF level consistently maintained somewhat above 5% will allow sufficient functioning of the hemostatic apparatus to control hemorrhage in many instances." It will be noted that AHF levels in this range were reached and maintained for varying periods of time in 3 of the 4 experiments shown in Fig. 1. With potent preparations of AHF, these modes of administration appear feasible and perhaps desirable.

AHF may appear in relatively large amounts in the plasma after extravascular injection. The reverse situation is to be expected: in time, some intravenously injected AHF should be redistributed to the extravascular spaces. This process may have been responsible, in part, for the different rates of disappearance of plasma AHF observed in the course of experiments of the type illustrated by Fig. 2. The rapid disappearance of AHF from the peak levels attained immediately after intravenous injection accords with previous observations in this laboratory(1,2) and could be mainly a manifestation of redistribution. Contrasted with this is the slow disappearance of AHF after time had been available for equilibration—22% to 11% in 23 hours. Depending on the extent to which equilibration between plasma and extravascular spaces had actually taken place, the AHF half-life would appear to be at least 23 hours.

Summary. 1. The plasma tide of AHF was measured after subcutaneous and intramuscular injections of bovine AHF fractions into hemophilic dogs. Better results were obtained with intramuscular than with subcutaneous administration; higher plasma AHF levels were maintained for longer times. Citrate in adequate concentration in the AHF solutions appeared to have an adjuvant action. 2. The rate of fall off of plasma AHF was followed after moderate levels of this clotting factor had been maintained for several hours. It is suggested that the biologic half-life of AHF in the dog may be at least 23 hours.

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Delayed Nidation in the Rat Induced by Progesterone.* (23418)

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Delayed implantation of blastocysts occurs in many diverse forms of mammals such as the roe deer, armadillo, bear, and many of the Mustelidae(4), and in the laboratory rat and mouse if the female is nursing a sufficient number of young while pregnant(3,4). Numerous reports have been published describing experiments done in an attempt to prevent or shorten delay under these conditions, but relatively little investigation has been done to artificially delay nidation in non-lactating females. The object of these studies was to determine whether nidation could be delayed by altering the normal hormonal relationship during early pregnancy in the laboratory rat.

Materials and methods. Mature female rats of the Sprague-Dawley strain were caged with mature males and the day of insemination determined by the presence of sperm in vaginal smears taken between 8:00 and 10:30 a.m. daily. The day that sperm were found was designated as Day 1 of pregnancy. The rats were maintained on Rockland rat diet and tap water. Surgical procedures were done under ether anesthesia and semi-sterile conditions. Laparotomies and ovariectomies were performed through dorso-lateral incisions. The ovaries were removed by slitting the ovarian bursa and by ligating the mesovarium, great care being taken to traumatize the oviduct as little as possible. Complete and partial hysterectomies were done through mid-ventral incisions. Rats were hypophysectomized by the parapharyngeal approach.

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16 mg of progesterone in corn oil were injected daily from the 2nd or 3rd day after breeding to the 9th day in the experiment designed to determine whether large doses of progesterone would delay implantation of embryos in intact normal female rats. All rats were laparotomized on the 8th day and their uteri were examined for the presence of implantation sites. Length of gestation and number of pups born was noted for all rats.

Since the data just described were negative as far as delaying implantation was concerned, a second type of experiment was done in which rats were ovariectomized 45-60 or 69-84 hrs after sperm were found. These rats were injected daily with 4 mg of progesterone in oil for variable periods from the 2nd-50th day, after which time they were injected with 4 mg of progesterone and 1 μg of estrone in oil per day for 10-16 days, and autopsied on the last day that estrone was injected.

All of the rats were laparotomized on the 8th day or on the day before estrone treatment was begun. If nidation had not occurred by that time the animals were laparotomized 5 days later to determine approximate time of implantation. Implantation was considered to have occurred if sites were found. Most of the rats were treated for 5-11 days after implantation had been determined at which time they were killed and autopsied.

Some of the rats were unilaterally hysterectomized or killed and the uteri flushed with either 0.9% saline or modified Krebs' solution and the flushings examined for the presence of blastocysts.

The results of the experiments just described demonstrated that removal of the ovaries followed by progesterone treatment will delay implantation of blastocysts for many days. It then became of interest to determine whether hypophysectomy would substitute for ovariectomy. Rats were hypophysectomized 22-33 or 44-57 hrs after sperm were found in the vagina and injected with 4 mg of progesterone per day from the 2nd-8th or 9th day and thereafter with 4 mg of progesterone and 1 μg of estrone from the 9th or 10th day to the 20th or 24th day. Three types of control animals were used for comparison with the results obtained in the experiments described above. All animals were ovariectomized 45-60 hr after finding sperm and one group of these was not injected, but was autopsied on the 5th and 21st days of pregnancy. Another group of ovariectomized pregnant females was not treated for 8 days but was injected thereafter with 4 mg of progesterone and 1 μg of estrone per day for variable times from the 9th to the 21st day, and autopsied on the last day of treatment. A third group was injected with 4 mg of progesterone and 1 μg of estrone per day from the 2nd-20th day and autopsied late on the 20th day.

Results. Delayed implantation of embryos was not observed in any of the intact rats given 16 mg of progesterone per day from the 2nd or 3rd day to the 9th day after insemination. Apparently the treatment had little or no effect on length of gestation and viability of the fetuses. These results are in agreement with those reported by Sammelwitz, *et al.*(8) who administered 4, 6, or 8 mg of progesterone daily from the day after mating to the 19th day in the rat.

Nidation of blastocysts was not consistently delayed in rats ovariectomized 69-84 hrs after insemination and given progesterone; embryos implanted in some of the females before injection of estrone was begun.

In the rats that were ovariectomized 45 to 60 hrs after breeding and injected with 4 mg of progesterone per day, implantation of blastocysts was delayed as long as the progesterone treatment was continued (Table I). In the majority of these rats implantation occurred within 5 days after administration of 1 μg of estrone per day was given in addition to the 4 mg of progesterone, which is essentially the treatment that Lyons used to maintain pregnancy from the 7th day to the 22nd and 23rd day in hypophysectomized-ovariectomized rats(7). These results are in con-

TABLE I. Effect of Treatment on Nidation.

Treatment	Day estrone begun	No. females failing to implant‖	No. females implanted		Estimated delay in nidation (days)
			Before estrone begun	Within 5 days after estrone treat. begun	
None	— (2*)	2	0	0	—
4 mg progest./day from 9th day	9 (6*)	6	0	0	—
4 mg progest./day from 2nd day	2 (6*)	0	—	6	0
Idem	7 (1*)	0	0	1	1
”	9 (10*)	1	0	9	3
”	10 (1*)	0	0	1	4
”	14 (6*)	3	0	3	8
”	19 (6*)	3	0	3	13
”	24 (2*)	0	0	2	18
”	29 (4*)	3	0	1	23
”	41 (3*)	1	1	1	35
”	51 (1*)	0	0	1	45
”	9 (2†)	0	2	0	0
”	11 (2†)	1	0	1 killed on 10th day; blastocysts recovered	5
”	14 (2†)	0	2	0	0
”	15 (1†)	0	1	0	0
”	9 (2‡)	0	0	2	3
”	10 (1‡)	0	1	0	0
”	9 (9§)	0	3¶	6	3

* No. animals—ovariectomized 45 to 60 hr after breeding.
† ” ” — ” 69 to 84 ” ” ” .
‡ ” ” —hypophysectomized 44-57 hr ” ” .
§ ” ” — ” 20-33 ” ” ” .
‖ As evidenced by laparotomies 1 day before and 5 days after beginning estrone treatment and at autopsy 5 to 68 days after breeding.
¶ Hypophysectomy not complete in 2 of these animals.

trast to those obtained with females which were ovariectomized-untreated and with those which were ovariectomized-untreated to the 9th day then injected with 4 mg of progesterone and 1 μg of estrone per day. As the data in Table I demonstrate implantation did not occur in any of these animals. Although no attempt was made to recover blastocysts from the uteri, it is assumed that the embryos in these rats died before implanting.

Ovariectomized females that were injected daily with 4 mg of progesterone and 1 μg of estrone, beginning on the day before ovariectomy, had embryos apparently implanted at the normal time (assumed to be the 6th day (5)).

Implantation occurred before the 8th or 9th day in only 4 of the 12 hypophysectomized females given 4 mg progesterone per day. In 2 of these 4 animals hypophysectomy was incomplete.

It is obvious from a study of the data in Tables I and II that even with the most successful treatments delayed implantation or recovery of blastocysts is not always accomplished. The reason for the variation is not precisely known, but it seems reasonable that inherent variations in time of ovulation, transport of embryos through the oviduct, and viability of embryos might account for most of the failures.

It is apparent that progesterone in the amounts used in this experiment will prevent nidation of blastocysts for prolonged periods of time up to approximately 45 days, and in such a state that they are capable of implanting if 1 μg of estrone is injected with the progesterone. This delay is, to the best of the authors' knowledge, approximately 28 days greater than the maximum reported for lactating rats(9).

The results obtained with progesterone and estrone in causing nidation are in essential agreement with those of Weichert(9) and

TABLE II. Recovery of Blastocysts.

Day uterus flushed	No. blastocysts recovered	Implant. in remaining uterine horn (day)		No. animals exhibiting implantation	No. animals failing to implant
		After	Before		
6 (1*)	0	6	11	1	0
8 (4*)	8	8	13	3	1
9 (1*)	1	9	14	1	0
13 (2*)	0	13	18	1	1
28 (2*)	3	—	—	0	2
40 (1*)	0	40	45	1	0
§ 8 (1†)	0	—	8	1	0
10 (2†)	5	—	—	0‖	1
8 (1‡)	5	8	13	1	0

Note: All animals inj. with 4 mg progest./day from 2nd day through the day of flushing, after which they were inj. with 4 mg progest. and 1 μg estrone/day.

* No. animals—ovariectomized 45 to 60 hr after breeding.

† " " — " 69 to 84 " " " .

‡ " " —hypophysectomized 20-33 hr " " .

§ Implantation at time of flushing.

‖ One animal killed on 10th day.

Krehbiel(6) for the rat, and those of Whitten in the mouse(10) who shortened delayed implantation by the administration of estrogen to pregnant lactating females.

While our studies were underway Canivenc and Laffargue(1) reported that they could delay implantation in rats ovariectomized on the 4th day after mating by injecting them intramuscularly with 5 and 10 mg of progesterone per day. However, we do not consistently obtain delayed implantation in rats ovariectomized on the 4th day and given 4 mg of progesterone (Table I). Chambon, as reported by Courrier(2), caused delayed implantation in rats ovariectomized 1 day after mating and given 0.25 mg of progesterone per day, but implantation occurred at the normal time if 0.50 mg progesterone per day was given. The delay caused by 0.25 mg progesterone per day treatment could be prevented if 0.16 μg of estradiol-17-beta was administered simultaneously with the progesterone. These data suggest that optimum conditions for implantation of embryos in rats are dependent upon the synergistic action of progesterone and estrogen. It seems probable on the basis of the information available that the quantitative aspects of this relationship must be formulated both in absolute and relative terms, and that many physiological factors participate in determining what this relationship will be.

A comparative study of blastocysts recovered as long as 28 days after mating with those obtained 8 days post-insemination demonstrates that both groups are in the inner cell mass stage, and that the older ones show little, if any, advance in development beyond that seen in the younger ones. Recovery of blastocysts from one horn of the uterus during progesterone treatment and subsequent implantation of the embryos in the other horn induced by progesterone-estrone provides strong circumstantial evidence that the blastocysts are in the uterus in a viable, but resting condition during progesterone treatment.

It is not known whether failure of the blastocysts to implant when progesterone alone is given is due to a defect in the embryo or in the uterus. Preliminary data which we have obtained, however, demonstrate that the endometria of the sterile horns of these rats do respond to artificial trauma by production of placentoma at a time blastocysts in the other horns are not implanted. The results of this and other experiments concerned with hormonal regulation of early rat development will be described later.

Summary. Nidation of embryos was delayed from 1 to 45 days in rats ovariectomized on the 3rd day after mating, by subcutaneous injection of 4 mg of progesterone per day. Implantation was delayed until such time as 1 μg of estrone per day was given in addition

to the progesterone. Recovery of blastocysts from one horn of the uterus during progesterone treatment and subsequent implantation of the embryos in the other horn induced by the progesterone-estrogen indicates that the embryos are maintained in the uterus in a viable, but resting condition during progesterone treatment. If rats were ovariectomized on the 3rd day and untreated for 8 days, nidation of embryos was not observed when progesterone and estrogen was initiated on the 9th day and given for the next 5 to 12 days. Rats ovariectomized on the 4th day after mating were variable in their response to 4 mg of progesterone, some exhibited delay others implanted at the normal time. Delayed nidation up to at least 3 days was obtained in hypophysectomized rats when they were injected only with 4 mg progesterone per day.

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Effect of Starvation on Sulfide Oxidizing System in Rat Liver.* (23419)

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In mammals, hydrogen sulfide is produced both by the microorganisms of the intestine (1) and by the body tissues from cysteine (2). The ability of mammalian tissues to oxidize small quantities of sulfide to thiosulfate and sulfate has been repeatedly demonstrated(3,4,5) and may under normal physiological conditions protect the tissue from sulfide poisoning.

In a recent study designed to elucidate the mechanism of this oxidation and the nature of the catalytic system involved(6), our research was initially complicated because of the great differences in ability of rat liver extracts from different animals to oxidize sulfide to thiosulfate. These differences were not eliminated by rigidly standardizing preparatory technics, age, sex, the commercial source of the rats, and method of sacrificing. It was noted, however, that rats sacrificed immediately after arrival from commercial sources (and in transit for some time with little food), and those used in experiments immediately following weekends (during which the feed in the rat colony had run low) almost always contained livers whose sulfide oxidizing capacity was high. Consequently, the effect of inanition on sulfide oxidation in rat liver was investigated.

Materials and methods. Young adult male rats of the Wistar strain ranging in weight from 200 to 250 g were used. They were maintained (unless otherwise specified) on Purina rat chow. They were sacrificed by decapitation, their livers exsanguinated *in situ* and an extract of the livers prepared. These procedures are described elsewhere in greater detail(6). The extracts were incu-

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TABLE I. Effect of Starvation on Sulfide Oxidizing System in Rat Liver (Summary of 14 Pair Trials).

S_2O_3 formation	Starved (20 to 48 hr)	Fed (control)	Difference (S − F)
Specific activity, μM/mg protein†/min.	.29 ± .04*	.095 ± .02	.20 ± .03
Total activity, μM/total protein†/min.	173.0 ± 15	91.2 ± 10	81.8 ± 11

* ± stand. error of mean.

† Protein determinations by method of Lowry *et al.*(13).

bated in phosphate buffer pH 7.3 with sulfide substrate in closed vessels. The thiosulfate formed was measured iodometrically(7) and enzymatically with rhodanese(8) as well as identified qualitatively by other chemical tests(7). The assay system as well as the assay procedure have been described(6). Cysteine desulfhydrase of the extracts was measured by a modification of the methods described by Smythe(9) and Almy(10) as follows: 2 ml of the liver extract in phosphate buffer were placed into the main compartment of a 2 armed Warburg vessel; 0.2 ml of 0.1 M L-cysteine HCl, 0.1 ml of 0.125 M. DPN and 0.1 ml of 0.125 M. α-ketoglutarate placed in the sidearms, together with sufficient Na_2CO_3 to bring the pH of the complete mixture to 7.6. The hydrogen sulfide generated upon incubation of the closed anaerobic system at 37°C, was absorbed by 0.2 ml of M. zinc acetate placed into a removable centerwell glass insert. After incubation (½ to 1½ hours), the insert was removed and the sulfide measured by the methylene blue method of Fogo and Popowsky (11).

Results. Effect of starvation. The effect of starvation on sulfide oxidizing ability of rat liver extracts is summarized in Table I. A total of 14 pairs of rats were matched with respect to age, weight, and rate of gain. One rat of each pair was fed while the other was starved for from 24 to 48 hours before sacrificing. At the time of autopsy every fed rat had food both in its stomach and gut. In every pair of rats the liver extract of the starved animal oxidized sulfide to thiosulfate 2 to 9 times more rapidly than the fed control. The average specific activity (per mg protein) of sulfide oxidation in the livers of starved rats was 3 times greater than in the fed controls. Starvation also doubled average total activity in the rat livers despite considerably lower liver weights for this group. The increased activity represented primarily, but not exclusively, the heat labile sulfide oxidase system(6). Statistical analysis(12) both on the basis of pair trials and group trials confirmed that the above differences in sulfide oxidizing ability of rat liver extracts from starved and fed animals was highly significant.

These differences became enhanced by incubating the extracts for longer periods of time. Fig. 1 shows that while the starved rat liver extract oxidized sulfide to thiosulfate at a linear rate for a considerable period of time, the fed rat liver extract, after some slight initial activity became incapable of oxidizing sulfide to thiosulfate at an appreciable rate.

Mechanism of activation. The mechanism

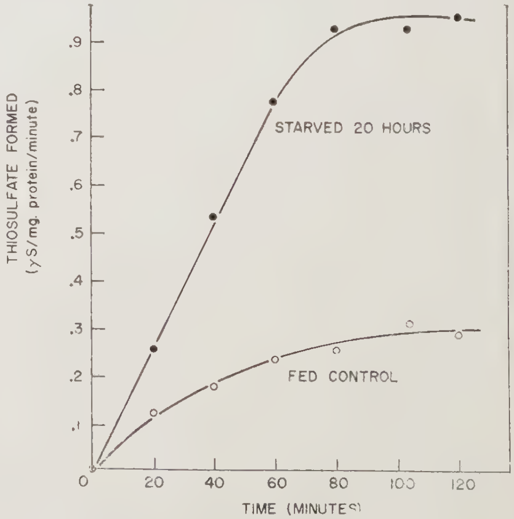

FIG. 1. Changes in activity with incubation time: A comparison. Activities plotted represent total activity including both heat labile and heat stable components of system(6).

by which starvation activates this system is as yet unknown. Excepting those cases in which food intake became affected, the feeding of synthetic diets high in cysteine, methionine, sulfur or sulfate did not enhance the capacity of rat liver to oxidize sulfide to thiosulfate. Intraperitoneal injection of sodium sulfide (up to lethal doses) and of cortisone (up to 7.5 mg/rat/36 hrs) did not elevate the sulfide oxidizing capacity of rat liver.

The feeding of diets high in sulfur-containing proteins (lactalbumin) gave equivocal results depending apparently upon the commercial source of the protein. The results suggested that rate and capacity for oxidizing sulfide to thiosulfate was not affected by the actual protein level of the diet but by some contaminating nutritional factor.

No systematic correlation could be demonstrated between increased rate of sulfide oxidation and cysteine desulfhydrase activity. However, addition of α-ketoglutarate and to a lesser extent DPN(2) to the reaction mixture, enhanced cysteine desulfhydrase activity so that this system under suitable pH conditions would not constitute the rate limiting step in oxidation of the sulfur of cysteine via sulfide to thiosulfate.

The possibility that activation of the sulfide oxidizing system could proceed through removal of an inhibitor was tested. When extracts of rat livers from starved rats whose sulfide oxidase specific activity was elevated 5-fold, were mixed with rat liver extracts from fed control animals, no inhibitory influence of the extract of the fed controls upon the activity of the active starved preparation could be demonstrated.

Discussion. The presence of catabolic systems in mammalian tissue, whose activity is elevated during periods of starvation, has been reported(14) and in the case of arginase this phenomenon could not be simulated by high substrate levels(15). The properties of the sulfide oxidizing system appear to be similar to the latter. The elevation of exogenous substrate either directly by injecting sulfide solutions or indirectly through inclusion of high levels of α-amino acids (in free form or in the protein of the diet) was ineffective in raising sulfide oxidase levels in the rat liver. Similarly, the elevation of endogenous substrate by accelerating gluconeogenesis through cortisone injections, was found to be ineffective.

The elevated rate of thiosulfate formation in liver extracts of starved rats does, however, correspond to Fromageot's and Royer's observation(16) that in intact rats starvation increased urinary excretion of thiosulfate. The feeding experiments suggest the possibility that elevation of some limiting closely bound cofactor for the system is responsible for the increased sulfide oxidation in starved animals.

Summary. A system in rat liver which oxidizes sulfide to thiosulfate is significantly elevated upon starvation, both in total activity and specific activity. This increase could not be induced *in vivo* by elevated substrate levels, and was not due to the loss of an inhibitor. Cysteine desulfhydrase levels in rat liver were not materially changed by starvation. These results have been discussed in the light of other findings.

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Action of Vitamin A on the Skin Following Intracutaneous Injection. (23420)

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Recent clinical reports (Steinberg(1,2), Mullens(3)) indicate that the human wart papilloma and certain types of helomata may be dramatically resolved by injection of vit. A palmitate directly into the lesion. These findings are unexpected since attempts to treat these lesions with vit. A by systemic administration have given equivocal results(4). The experiments described below were therefore undertaken to study the effect of vit. A on normal epidermis when administered by the intracutaneous route.

Methods. A commercial vit. A palmitate solution (Keramin Injection) especially formulated for injection of skin lesions was used throughout. This solution has an activity of 50,000 U.S.P. units per cc in a vehicle consisting in 11% polysorbate 80, U.S.P., 0.25% citrate-phosphate buffer, pH 7, with 0.5% chlorobutanol, 0.4% dl-alpha tocopherol and 0.03% butylated hydroxyanisole as preservatives and antioxidants. A sterile solution containing all ingredients except vit. A was used for control injections.

New Zealand white male rabbits weighing 2-4 kg were used in all experiments. The dorsal thoracic and abdominal area of each rabbit was clipped closely, care being taken to avoid cutting the skin, and the entire surface was washed with 70% ethanol. A volume of 0.03 cc of the solution to be tested was injected intracutaneously by means of a 0.25 cc tuberculin syringe and a sharp, 27 gauge, ½ inch, regular point hypodermic needle. Injections were made only after the bevel of the needle was seen to be just below the surface. The needle was left in the skin for about ½ minute after injection to minimize leakage from the point of puncture. Pairs of injections were made on either side of the mid-dorsal line but never over a bony prominence. When vit. A was injected on the left side, vehicle was injected on the right side at the same level and vice versa. Sites of injection were marked with indelible pencil. The animals were sacrificed always in the late afternoon by concussion 1, 2, 4, 7, 14, 21 and 28 days after injection. In all cases, a strip of skin at the site of injection approximately 40 x 4 mm was excised for histology so as to contain a central injected region and uninjected skin on either side. These latter areas served as additional controls. Tissues were fixed in Bouin's fluid, sectioned through the long axis at 8 μ and stained with Mayer's acid alum hematoxylin and eosin. The thickness of the epithelial layer with the corneum excluded was estimated with an ocular micrometer at 450 X magnification. Measurements were made in the center of each section as well as at points approximately one-tenth of the length in from either edge (uninjected controls). Only areas between the hair follicles were used for evaluation. Mitotic figure counts were made in the regions where epithelial thickness was measured. Two hundred and fifty consecutive nuclei in the basal and spinous* layers were counted for the areas corresponding to vit. A injection, vehicle injection and normal uninjected controls.

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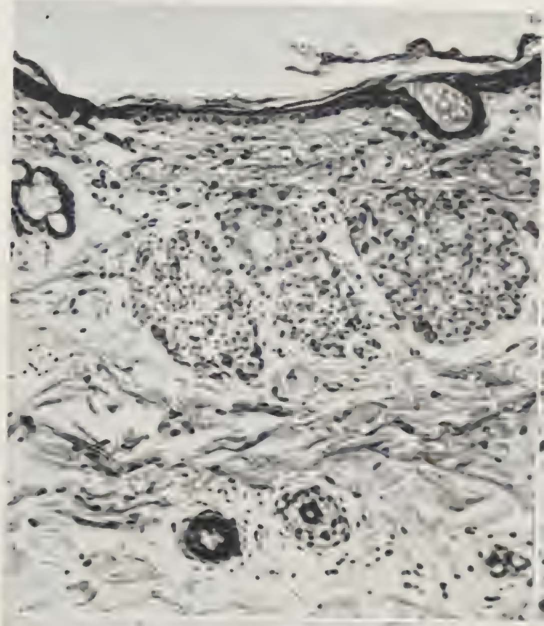
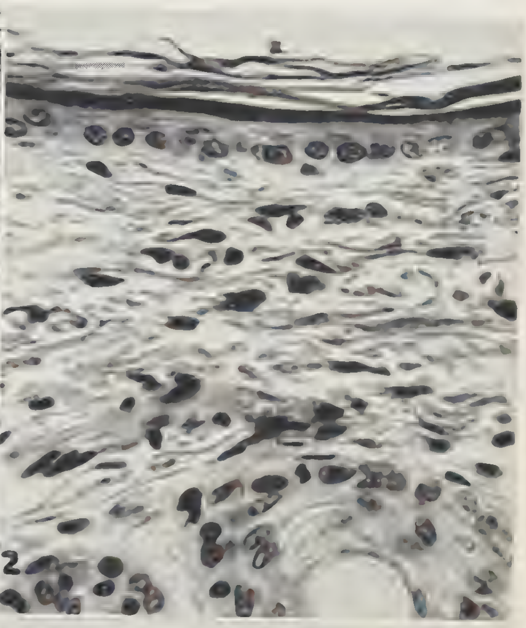
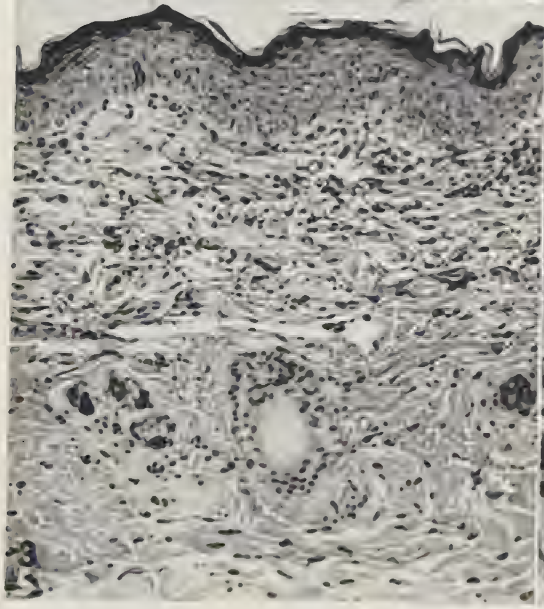
* The term spinous layer used in this paper refers to the cells in the germinative layer other than the basal layer even though no "prickle cells" as such can be found in the skin.

Results. On gross examination of the living animals, a mild erythema is seen after vit. A injection which appears in 24 hours and lasts several days. Vehicle injections give rise to a less intense response of somewhat shorter duration.







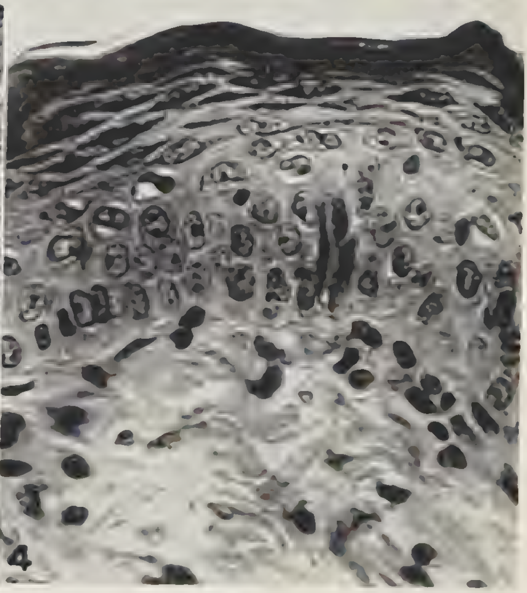


FIG. 1. Section of rabbit skin 7 days after intracut. inj. of vehicle control showing essentially normal skin. H & E × 128.

FIG. 2. Higher magnification of Fig. 1. The epidermis is 1-2 cell layers thick and the corneum diffuse. H & E × 480.

FIG. 3. Section of rabbit skin 7 days after intracut. inj. of aqueous vit. A solution. Epidermis has thickened considerably. H & E × 128.

FIG. 4. Higher magnification of Fig. 3. Nuclei in the basal layer are larger than in controls. Cells in various stages of differentiation are present including several layers of granular cells. Corneum is dense, compact and closely adherent to epidermis. H & E × 480.

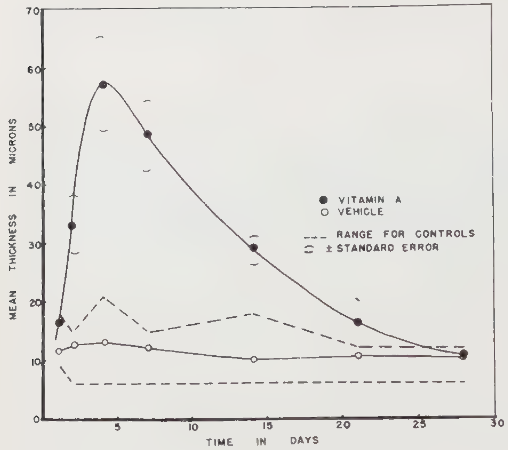


FIG. 5. Effect of a single inj. of vit. A on thickness of rabbit epidermis excluding corneum. Range only is given for the controls because of the relatively high measurement error in layers only a few cells thick.

Microscopic sections show minor changes in the dermis suggesting slight irritation. A dramatic change in the architecture of the epidermis occurs after vit. A but not after vehicle (Fig. 1 to 4). These changes may be summarized as follows: (a) *Epidermal thickness*: The epidermis (excluding corneum) increases about 5 fold in thickness within 4 days and gradually returns to its original dimensions about 2 weeks later (Fig. 5). Thickness of epidermis is unchanged by vehicle injections. (b) *Cyto-architecture*: Normal rabbit epidermis (or epidermis after injection of vehicle) is rarely more than 2 cells thick and consists in a basal layer over which more highly differentiated cells are distributed sporadically. The corneum is diffuse and basophilic. After vit. A injection the epithelium shows distinct layers of cells in various stages of maturation. Thus germinative, spinous and granular layers are clearly evident. The corneum is now thinner, denser and decidedly eosinophilic. No stratum lucidum can be seen in either control or treated tissues. It is of interest that the nuclei in the germinative layer after vit. A injection are larger and younger in appearance than in control epithelium. The increase in thickness of the epidermis is due to an increase in both size and number of cells. Formation of rete pegs has not been observed. (c) *Occurrence of mitosis*: In order to determine whether vit. A increases rate of cell division or slows down rate of maturation and death of cells, counts were made of the number of nuclei undergoing mitosis in the basal layer and spinous layer of tissues taken 2, 4 and 7 days after injection. As can be seen in the table, there is no striking difference between the number of nuclei undergoing mitosis in any group. However, minor differences in mitotic rate would hardly be detected because of the relatively small number of nuclei counted. (d) *Distribution of cell types*: It will be clear from the counts in the table that vit. A causes a change in the ratio of nuclei in the basal layer to the spinous layer from 9 to 1 to about 1 to 1.

Discussion. The effects of vit. A on the skin by topical application or systemic administration have been studied by others(5-8) and somewhat similar, but less dramatic and reproducible changes have been reported. It seems most likely that this is due to the comparatively low concentration which can reach the cells by these routes since still more profound changes in the epidermis have been reported by Fell and Mellanby(9) who found that very high concentrations of the vitamin, such as can be achieved only in tissue culture, will suppress keratinization altogether and change the epidermis to mucous secreting epithelium. After systemic administration (oral, intramuscular, intravenous, subcutaneous) storage of vitamin in the liver would operate

TABLE I. Occurrence of Mitosis in Rabbit Epidermis after Injection of Vit. A.

Time after inj., days	No. of slides counted	Mean % of various nuclei observed out of 250 cells counted: Basal layer, Normal nuclei	Basal layer, Mitotic nuclei	Spinous layer, Normal nuclei	Spinous layer, Mitotic nuclei
		Vitamin A			
2	8	62.5	.4	37.1	0
4	4	46.4	0	53.6	0
7	12	46.7	.2	53.0	.03
		Vehicle			
2	8	89.1	.3	10.6	0
4	4	88.3	.1	11.6	0
7	12	89.2	.1	10.7	0
		Uninj. controls			
2	8	90.0	.3	9.7	0
4	4	88.8	0	11.2	0
7	12	88.0	.2	11.8	0

against attaining a high concentration in the skin. Topical application would also appear to be an unreliable approach to the epidermis especially in the case of human skin lesions since the conditions of interest present an unusually thick keratin barrier. Furthermore, the absence of sebaceous glands in plantar skin would almost preclude penetration of vit. A at this most common site of verrucae and other hyperkeratoses since the sebaceous glands form the normal pathway for absorption of the vitamin. Intraepidermal injection of vit. A is, of course, a practical impossibility, so that, intracutaneous injection would appear to be the only efficient method of introducing vit. A at the site of an epidermal lesion.

There seems to be only one possible interpretation for the results here reported. The presence of large (hence young appearing) nuclei in the basal and spinous cell layers of the epidermis coupled with a normal rate of mitosis, indicates that the physiologic sequence of maturation and death of these cells has been slowed. Therefore, cells of all types have accumulated and less keratin has been laid down. If these changes occur also in human skin it is reasonable to suppose that injection of vit. A at the site of an heloma would reverse the pathologic process since this lesion shows a central area of atrophy of the epidermis with an overlying plug of horny material. The mechanism of action in the case of verruca, a proliferative virus disease, is still obscure and future work will be directed toward its clarification.

Summary. It has been found that a single intracutaneous injection of vit. A in the rabbit causes an increase in thickness of the epidermis (excluding corneum) which is due to an increase in both number and size of the cells in the germinative and granular layers. The corneum becomes thinner and denser. The occurrence of large, young appearing nuclei in the absence of an increase in mitotic rate indicates that the maturation process is slowed.

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Resistance of Human Gingival Collagen to Human Gingival Bacteria.[*]

(23421)

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Since breakdown of the collagen fibers that anchor the teeth is a principal feature of periodontal disease, the demonstration that microorganisms present in the gingival sulcus or periodontal pocket produce collagenases assumes importance. Investigators using collagen paper or azocoll as substrates(1,2) have reported isolation of oral and gingival organisms that exhibited either collagenase-like or true collagenase activity. However, other proteolytic enzymes, such as trypsin, are capable

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[*] This investigation was aided by a contract between Office of Surgeon General, Department of Army, and University of Rochester.

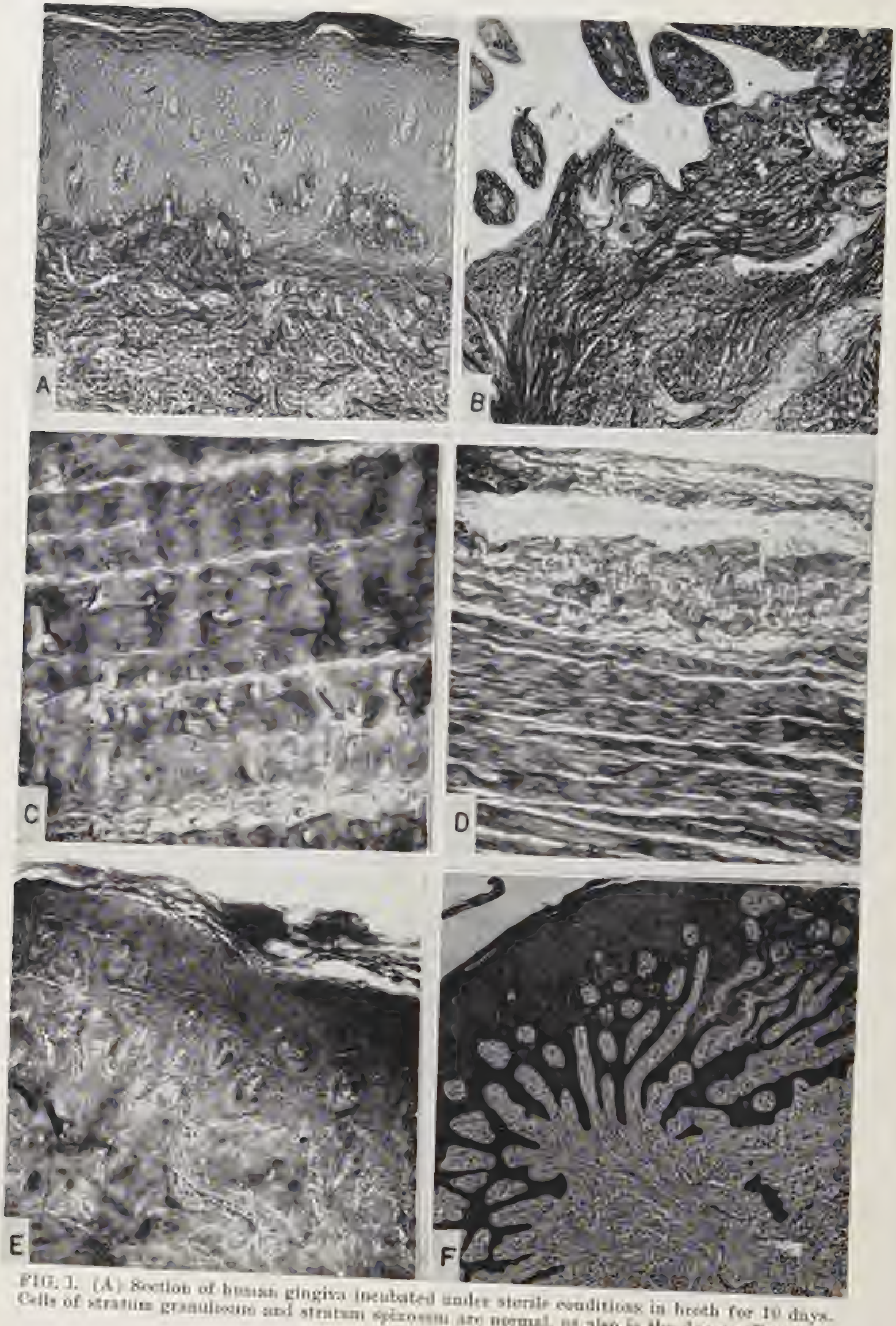

FIG. 1. (A) Section of human gingiva incubated under sterile conditions in broth for 10 days. Cells of stratum granulosum and stratum spinosum are normal, as also is the dense collagenous

of degrading both collagen paper and azocoll, *i.e.,* denatured collagen. In an attempt to simulate as nearly as possible *in vivo* conditions, Schultz-Haudt and Scherp(3) used as substrate fresh bovine Achilles' tendon stored at –20°C and sterilized before use with ethylene oxide vapor for 18 hours at room temperature. Such treated tissue was deemed unaltered, since it appeared normal histologically when stained with hematoxylin and eosin, and resisted digestion by crystalline trypsin. The authors concluded that oral bacterial collagenases may indeed play a significant role in the breakdown of periodontal collagen. A similar technic has been used by Roth and Myers(4). Except for the occasional isolation of a *Clostridium* species, organisms able to lyse unaltered collagen were not found in cultures of the contents of either normal gingival sulci or periodontal pockets. The present experiments were undertaken to extend the previous work(3) to human gingival tissue.

Methods. Fresh human gingival tissue, obtained, without pretreatment, by gingivectomy on patients suffering from periodontitis simplex, was placed immediately in 10 ml of trypticase-casein-yeast-extract broth and incubated aerobically for 10 days, after which time the tissue and bacterial growth were removed by centrifugation and the supernatant fluid was analyzed for hydroxyproline, as a measure of collagenolysis(3). Representative samples of gingival tissue in each test varied in dry weight from 5 to 20 mg and contained from 30 to more than 160 μg of hydroxyproline. The minimum significant increase of hydroxyproline was considered to be 2 μg per ml of supernate, when compared to control broth cultures of the bacteria indigenous to the original tissue. Anaerobic incubation of the tests did not materially affect the results and was, therefore, employed only in the first experiments. In tests of samples from 21 patients, no collagenolysis could be detected (Table I). This resistance of the gingival collagen to digestion was confirmed by histological examination. After the supernate had been removed for hydroxyproline analysis, each piece of gingival tissue was fixed in formol-saline and embedded in paraffin. Sections were stained respectively with hematoxylin and eosin and by the Mallory-azan technic (Heidenhain's modification). For control, a sample of each specimen had been incubated for 10 days in sterile conditions, maintained by 200 i.u. of penicillin and 200 μg of streptomycin per ml. Fig. 1A shows that only slight cellular autolysis occurred in the control specimen, as seen in the epithelium and inflammatory infiltration of the underlying connective tissue. In contrast, Fig. 1B illustrates the complete disorganization of all tissue components, other than collagen and reticulin, produced by incubation with the gingival bacteria. The connective tissue in these specimens stained normally with the Mallory-azan method and was microscopically unaltered, although no fibroblasts were observed. As a control in each experiment, a sample of bovine Achilles' tendon, which had been stored in the frozen state and sterilized by ethylene oxide, was incubated in broth with the biota from the respective sample of gingival tissue. Collagenolysis was demonstrable in 15 of the 21 tests. Furthermore, the collagen of gingival tissue and tendon, sterilized with antibiotics, was shown to be digested by filtrates of broth cultures of *Clostridium histolyticum, i.e.,* true collagenase

dermis, the cellular infiltration of which is clearly visible. There is slight "loosening" of the stratum spinosum cells immediately adjacent to the stratum germinativum. (B) Section of human gingiva incubated for 10 days in broth with its indigenous flora. Complete disintegration of all elements other than the dermal connective tissue is apparent. (C) Bovine Achilles' tendon stored at –20°C and treated with ethylene oxide vapor for 18 hr. The fibroblasts are not well differentiated and the collagenous fibers appear homogeneous and swollen. (D) Bovine Achilles' tendon stored at –20°C. The fibroblasts are well differentiated and the collagen bundles appear compact. (E) Human gingival tissue treated with ethylene-oxide vapor for 18 hr. Homogenization is obvious. The dermal connective tissue appears "hyalinized" and merges with the stratum germinativum. Rete pegs can still be seen in the stratum spinosum. (F) Untreated human gingival tissue. All dermal and epidermal elements are clearly differentiated. (A) and (B), ×45, Mallory-azan stain; (C) and (D), ×75, Mallory-azan stain; (E) and (F), ×45, stained with hematoxylin and eosin.

TABLE I. Collagenolytic Activity of Human Gingival Bacteria on Human Gingival Tissue and Bovine Achilles' Tendon.

Series	Tissue	Treatment	Organisms*	No. positive / No. tests	Control tendon†
1	Gingiva	None	Indigenous (10 days)	0/21	15/21
	"	Antibiotic sterilization	*Cl. histolyticum* filtrate (3 days)	6/ 7	
	Tendon	*Idem*	*Idem*	1/ 1	
	Gingiva	None	Indigenous (6 wk)	3/ 5	5/ 5
2	Tendon	Freezing and ethylene oxide	Subgingival scrapings‡ (10 days)	8/11 (J.T.) 13/19 (S.–H.)	
3	Gingiva	None	Indigenous (10 days)	0/ 8	7/ 8
	"	Freezing and ethylene oxide	" "	5/ 8	

* Aerobic incubation.

† Stored in the frozen state, sterilized by ethylene oxide, and incubated with microbiota from respective untreated gingival tissue.

‡ From cases of chronic marginal gingivitis. J.T. denotes results of present experiments. S.–H. denotes previously reported results(3).

(Table I). Continued incubation of 5 randomly selected tests for 6 weeks increased the breakdown of tendon in all cases and liberated a small but significant amount of hydroxyproline-containing material in 3 of the 5 previously negative tests of untreated gingival tissue. Finally, we confirmed the results of Schultz-Haudt and Scherp(3) by showing that broth cultures of the crevicular microbiota from 8 of 11 subjects with localized chronic marginal gingivitis lysed the collagen of tendon that had been frozen, thawed, and sterilized by ethylene oxide (Table I). These results indicated that such treatment rendered collagen susceptible to bacterial proteases other than collagenase.

Results. To test this conclusion, specimens of gingival tissue were divided into halves, one of which was incubated immediately in a tube of broth with its indigenous microbiota. The other was placed in a sterile tube, held at –20°C for 72 hours, thawed, and sterilized by exposure to ethylene oxide vapor for 18 hours; broth was added aseptically and inoculated with the growth from the first tube. The sterilized halves underwent collagenolysis in 5 of 8 tests, compared with 7 of 8 tendon controls, whereas none of the untreated halves did so (Table I). The alteration of the substrate in these experiments could be due to denaturation by freezing and thawing, chemical action of ethylene oxide, or a combination of these factors. These possibilities were explored in 15 additional experiments, in which the specimens of gingival tissue were divided into quarters, which were transferred to sterile tubes and treated as follows: (1) incubated immediately with its indigenous flora in 10 ml of broth added aseptically; (2) held at –20°C for 3 days; (3) sterilized by immediate exposure to ethylene oxide vapor for 12 hours; and (4) held at –20°C for 3 days, thawed, then sterilized by ethylene oxide vapor at room temperature for 12 hours. In 4 cases, sufficient tissue was available to provide a fifth portion, which was sterilized by immediate submersion in acetone at –8°C, transfer after 12 hours to a sterile tube, and evaporation of the residual acetone at 37°C for 12 hours(1). Each test included as a control a tube containing a sample of bovine Achilles' tendon, previously frozen, thawed, and sterilized by ethylene oxide. At the conclusion of the respective treatments to tubes 2 *et seq.*, broth was added aseptically, inoculated with the growth from tube 1 and incubated aerobically for 10 days.

Once again, collagenolysis of the untreated gingival tissue was not demonstrable (Table II). Freezing or treatment with ethylene oxide rendered the collagen slightly susceptible to digestion in a few cases, but the combination of these procedures was far more effective. Gingival collagen sterilized by ace-

TABLE II. Effect of Freezing or/and Ethylene Oxide on Susceptibility of Gingival Tissue to Collagenolysis by Indigenous Gingival Bacteria.

Tissue	Treatment	No. positive / No. tests*
Gingiva	None	0/15
”	Freezing	2/15
”	Ethylene oxide	4/15
”	Freezing & ethylene oxide	12/15
Bovine tendon	*Idem*	15/15
Gingiva	Acetone	0/ 4

* All cultures incubated aerobically for 10 days.

tone, however, resisted digestion. In this series, all of the control tests of tendon resulted in collagenolysis.

Alterations of collagen by freezing and ethylene oxide were demonstrated also by histological means. Fresh specimens of human gingival tissue and bovine Achilles' tendon were stored at –20°C for 3 days, thawed, sterilized with ethylene oxide vapor as described previously, fixed in formol-saline, and embedded in paraffin. Sections were stained with hematoxylin and eosin and by the Mallory-azan technic. The treated tendon stained with hematoxylin and eosin revealed no difference in staining properties, although under high power the collagen fibers appeared swollen and homogeneous with either stain (Fig. 1C), when compared to those of untreated tendon (Fig. 1D). Similarly treated and stained human gingival tissue, however, showed complete homogeneity of the connective tissue elements and loss of differentiation between the basal cell layer and the underlying connective tissue (Fig. 1E and F). The Mallory-azan technic revealed striking differences between the treated and untreated tissues. In both treated tendon and gingival tissue there was a loss of the blue stain characteristic of normal collagen. Instead, the orange G was largely retained throughout the dense collagenous elements of the tissues. Prolonged exposure to the anilin blue-orange G reagent (1½ hours or longer) in most cases reversed the staining of the connective tissue toward normal, although even after this time a slight background of orange G was still apparent. Also, the epithelium of the treated gingival tissue took the orange G much more intensely. On the other hand, cellular elements such as erythrocytes and inflammatory cells stained normally and so did the loose reticular fibers that surround the blood vessels, etc., and form the connective tissue support of the basement membrane of the gingival tissue. The observed changes were probably attributable to the action of ethylene oxide, for they were identical in gingival tissue treated with ethylene oxide without prior freezing (Fig. 1E).

Discussion. In 3 series of tests on specimens of untreated gingival tissue from 44 patients with sufficiently severe periodontitis simplex to warrant gingivectomy, the gingival collagen resisted digestion during a 10-day period of incubation with those members of the indigenous gingival microbiota that grew under the conditions of our experiments. After 6 weeks' incubation, 3 of 5 additional tests showed only slight collagenolysis. The discrepancy between these results and those previously reported(3) can be explained by the fact that the collagen becomes susceptible to proteases of the gingival microbiota when frozen, thawed, and sterilized by ethylene oxide, as in the prior study. However, it still resists crystalline trypsin(3).

The extreme resistance of native collagen to enzymatic degradation has been thoroughly documented. Thus, Neuman and Tytell(5) found that only *Clostridium perfringens, Cl. histolyticum,* and pepsin attacked all of the various collagenous substrates they tested and emphasized that the degree of enzymatic degradation was proportional to the degree of denaturation that the collagen had undergone during its preparation. MacLennan *et al.* (6), using sterile rabbit Achilles' tendon as a native collagenous substrate, could find evidence of collagenase production by only *Cl. perfringens* and *Cl. histolyticum.* Except for the rare occurrence of *Clostridium* species, the gingival microbiota has not been shown to produce a true collagenase. Consequently, a role for this enzyme in periodontal disease has not been substantiated. However, it is almost certain that many species of this microbiota did not grow under the conditions used and the possibility remains that these organisms, alone or in combination, can effect collagenolysis. There is ample evidence of oral bacterial proteases, other than collagenase, capable of attacking altered collagen(1,2,4,7). The slight collagenolysis observed after prolonged incubation of untreated gingival tissue with its indigenous microbiota may also be secondary to substrate alteration. It seems reasonable to suggest, therefore, that the loss of collagen fibers characteristic of periodontal disease results not from the primary action of microbial collagenase but from an alteration of the collagen which renders it digestible by other proteases of the resident crevicular flora, in the manner postulated by Sherry *et al.*(8). However, no mechanism has been demonstrated whereby such an alteration could occur *in vivo*.

Ethylene oxide has been recommended for sterilization of sundry materials and culture media and it has been generally assumed to be relatively benign. Judge and Pelczar(9) found that it had no detrimental chemical effect on a series of carbohydrates. Recent work by Windmueller *et al.*(10), however, shows that ethylene oxide renders casein nutritionally ineffective for rats. According to Fraenkel-Conrat(11), 1,2 epoxides reacted readily with the carboxyl and basic groups of proteins in aqueous solution. However, their binding capacities for an acid dye (orange G) were undiminished (*cf.* also Einbinder and Schubert(12) on the binding of dyes by collagen under different ionic conditions). This fact, and the absence of a comprehensive theory of the tri-acid staining methods for connective tissue make it difficult to rationalize the altered staining reactions observed in tissue treated with ethylene oxide. In any event, the net effect was to decrease the rate of displacement of orange G from the collagen by the less strongly acid anilin blue. Our results appear analogous to the recent observations of Fullmer and Lillie(13) who report altered staining reactions of collagen with orcein following blockade of polar groups of the collagen by dinitrofluorobenzene, benzoylation, methylation, and acetylation.

Summary. In 44 tests, in which untreated human gingival tissue from cases of periodontitis simplex was incubated for 10 days in broth with its indigenous microbiota, no breakdown of collagen could be detected either chemically or histologically. All other tissue elements, however, were stripped away. In concurrent controls, bovine Achilles' tendon, stored frozen and sterilized by ethylene oxide as in a previous investigation, was used as a substrate for the same microorganisms and underwent collagenolysis in 37 of 44 tests. Freezing, thawing, and sterilization of human gingival tissue with ethylene oxide rendered its collagen susceptible to degradation by its indigenous flora in 17 of 23 tests. This action of ethylene oxide was paralleled by a striking alteration of the reaction of the tissues to the Mallory-azan stain for connective tissue. The participation of true bacterial collagenases in periodontal disease has not yet been substantiated. Conclusions to the contrary have been based on the digestion of altered collagen by other proteases.

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Experimental Lathyrism in Mice Fed Diets Containing Sweet Peas or β-aminopropionitrile.* (23422)

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When β-aminopropionitrile (BAPN) or the seeds of *Lathyrus odoratus* (sweet pea) are fed to rats, an experimental lathyrism is produced which is characterized by skeletal and aortic changes(1-9). There are no reports in the literature concerning similar findings in mice. In fact, it is generally supposed that mice are unaffected by the toxic factor of sweet peas because Lewis and Schulert(10) reported that white mice showed no evidence of toxicity when fed diets containing 50% sweet peas for 17 weeks.

In a preliminary experiment, we fed a diet containing 50% sweet peas to 3 weanling mice. These mice grew normally and showed no obvious skeletal changes. After 4 weeks of feeding, however, 2 animals died suddenly from ruptured aortas. We have, therefore, studied the effect of feeding diets containing sweet peas or BAPN to albino mice to determine the difference in response, if any, between mice and rats to such diets.

Methods. Weanling, Swiss albino, male mice, from the mouse colony of the Department of Oncology, were used. The pea diets were made up as previously described(11). The BAPN-containing diets were prepared by drying a solution of synthetic BAPN hydrochloride on a weighed amount of milled Rockland rat stock diet. The BAPN used in these diets was synthesized by the method of Buc (12), converted to the hydrochloride, and crystallized from 95% alcohol. All diets were supplied *ad libitum*. Animals were weighed weekly and autopsied at death or at termination of experiment. For microscopic studies, tissues were fixed in 10% buffered formalin or alcoholic formalin (10% in 95% alcohol). They were dehydrated, cleared and embedded in paraffin. Six to 10 μ sections were stained with hematoxylin and eosin, or a modified Hornowski's combined elastic and Van Gieson method for connective tissues. Selected sections were stained with Lillie's modification of the McManus P.A.S. method.

Results. As indicated in Table I, when diets containing 50% sweet peas or 0.2% BAPN • HCl were fed, the mice grew normally. Higher levels of BAPN caused a depression in growth rate in proportion to the level of BAPN fed. Although many deaths occurred suddenly and without warning, on higher levels of BAPN death was sometimes preceded by weight loss.

After 3 weeks, mice receiving 50% sweet peas or 0.2% BAPN • HCl showed none of the symptoms of advanced experimental lathyrism observed in *all* rats consuming such diets for similar periods(3,4). Only after 4 to 7 weeks of feeding did a few of these mice develop palpable spinal curvatures. At autopsy, most animals in these groups had small but well-defined exostoses on the medial aspects of their femurs in contrast to rats which had grossly misshapen femurs in much shorter time intervals(3,4) (Fig. 1). Three of the 44 mice on these diets developed paraphymosis (a common occurrence in rats receiving such diets for similar periods). Twelve of the 44 mice had gross aneurysms of the aorta, 7 of which ruptured causing death of the animals. Deaths from aortic rupture occurred at varying times between the 14th and 68th day after beginning the experimental feedings.

Differences in composition of the sweet pea diets used by us and by Lewis and Schulert (10) would not seem to explain the complete resistance of their mice to the toxic factor. While increased levels of casein have been found to be partially protective against skeletal(3,13,14) as well as aortic lesions(15,16) in experiments with rats, in the present experiments a level of casein which was higher

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TABLE I. Incidence of Gross Aneurysms and Aortic Rupture in Mice Fed Sweet Peas or BAPN.

Exp.	No. of mice	Mean initial wt, g	Mean wt at 28 da., g	Diet		Deaths		Survivors killed		Total No. gross aneurysms
				%		No.	Cause	At days	Autopsy	
I	9	13.7	25.1	50 10	sweet pea casein	2	Ruptured aortas	51	2 aneurysms 5 normal	4
	9	14.1	24.2	.2	BAPN · HCl	1 1	" " Undetermined	52	1 aneurysm 6 normal	2
	6	13.6	24.9	50 10	edible pea casein	0		52	6 "	0
	7	12.4	23.1	Rockland stock		0		52	7 "	0
II	11	13.7	20.0	.5	BAPN · HCl	9	Ruptured aortas	76	2 "	9
	11	13.6	17.6	1.0	"	2 6	" " Undetermined*	76	3 "	5*
	11	13.6	24.7	Rockland stock		0		76	11 "	0
III	15	12.5	22.7	50 10	sweet pea casein	1 1	Ruptured aorta Undetermined	70	2 aneurysms 11 normal	3
	11	12.7	22.2	50 20	sweet pea casein	3 1	Ruptured aorta Undetermined	70	7 "	3

"Normal" signifies absence of gross aortic aneurysms.

* Three of the 6 animals which died from undetermined causes in this group had gross aneurysms.

than that used by Lewis and Schulert gave no apparent protection.

Only mice receiving 0.5% and 1% levels of BAPN • HCl showed skeletal changes comparable to those occurring in rats receiving much lower levels of BAPN (Fig. 1). Spinal curvatures varied from slight to very severe. A few animals became severely crippled. Some animals survived 10 weeks of feeding, however, without showing symptoms and showed only minor skeletal changes at autopsy. Skeletal effects were therefore much more variable in mice than in rats, all of which rapidly develop severe skeletal changes at much lower levels of BAPN.

Deaths in groups receiving the 2 higher levels of BAPN • HCl (17 of 22 mice) occurred between the 20th and 75th day of the experiment. On the 1% level, mice which died within the first 6 weeks of the feeding period had severely deformed femurs. These animals also showed a greatly increased vascularity in periarticular areas and an abnormal red color of the long bones. Paraphymosis occurred in 5 of 11 mice at each of these levels of BAPN. Even though extreme paraphymosis and severe crippling occurred in some animals, no instance of paralysis or urinary retention was seen in any of the groups. The latter 2 symptoms are common in experimental lathyrism in rats.

The incidence of aortic aneurysms was also increased by higher levels of BAPN. The incidence of gross aneurysms is given in the Table. Deaths from aortic rupture occurred in all experimental groups. No ruptured aortas or gross aneurysms were found in any mice receiving either the Rockland or the edible pea control diets. Of the total of 26 gross aneurysms distributed throughout the experimental groups, 24 were located in the thoracic aorta. The remaining 2 were found in the upper lumbar area of the abdominal aorta.

Aortic lesions. Microscopically, the *normal mouse aorta* is similar to that of the rat. The intima is very thin, consisting mostly of endothelium. The media forms about 5/6ths or more of the entire wall and is well defined, consisting essentially of wavy elastic fibers, smooth muscle, and a small amount of collagen with fibrocytes. The adventitia is less well defined, relatively thin, and composed almost entirely of collagenous connective tissue with small numbers of rather delicate elastic fibers occurring in the inner portion.

Although not all aortas from experimental animals were examined, *histopathologically* no

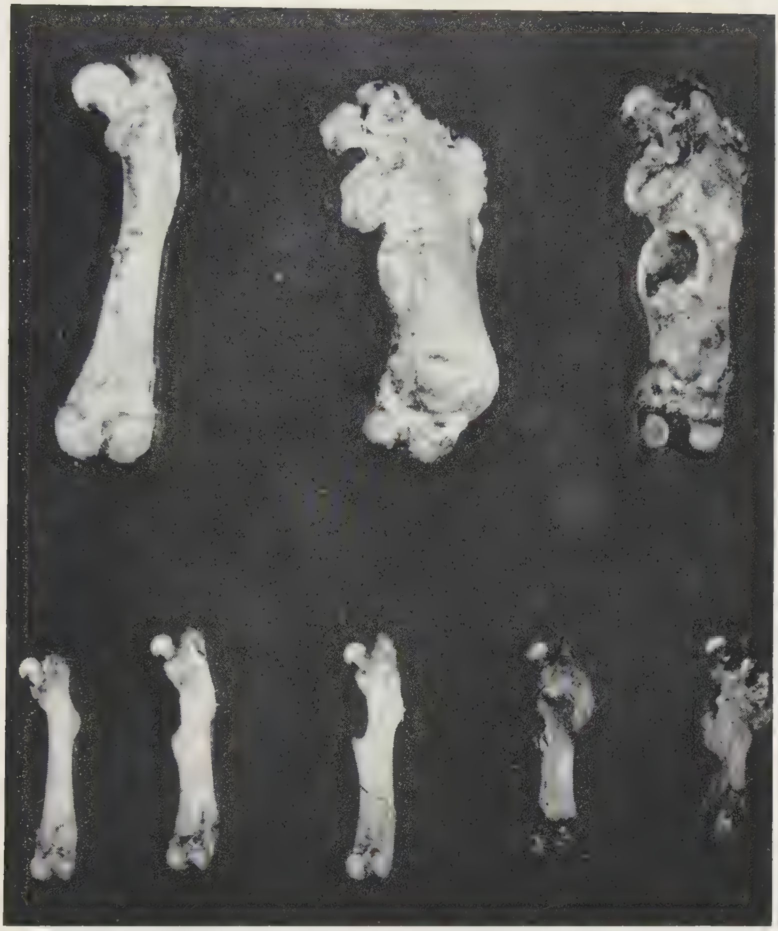

FIG. 1. Top row, rat femurs, left to right: normal; 50% sweet peas, 22 days; 0.25% BAPN · HCl, 15 days. Bottom row, mouse femurs, left to right: normal; 50% sweet peas, 50 days; 0.2% BAPN · HCl, 56 days; 0.5% BAPN · HCl, 28 days; 1% BAPN · HCl, 34 days. The rat femurs illustrate *typical* changes, the mouse femurs illustrate maximal changes in animals receiving above diets.

entirely normal aortas were found in any animals receiving sweet pea- or BAPN-containing diets.

No lesions of the *intima* were observed that appeared to be primary in nature. Proliferative changes were frequently observed but always appeared secondary to, or compensatory for, destructive lesions in the media. The early ones were quite cellular (endothelial and fibroblastic) and became more fibrous with increasing age of the lesion. In older aneurysms with thrombosis this intimal proliferation extended into the thrombus, aiding in its organization. Such organization was not extensive and seemed slow in its progress. Mural thrombi were observed only in cases with aneurysm formation. Recanalization of thrombi was not observed. Significant amounts of cholesterol or lipids were never observed.

Changes in the *media* seemed to be primary but their exact nature and sequence were often not readily apparent. The earliest change occurred between the elastic fibers and resulted in separation of these fibers. Initially the smooth muscle cells and fibrocytes between the elastic fibers seemed intact, indicating that the change was in the amount of ground substance. Whether this was simply an infusion of fluid, an edema, or a true increase in the amount of ground substance could not be determined by the methods employed. Following this, retrogressive changes were observed in the cells (myocytes and fibrocytes) between the elastic fibers. Varying numbers of these cells were destroyed resulting in a temporarily decreased cellularity of the media. The next change was in the elastic fibers and appeared to be a combination of swelling, fragmentation, and lysis which resulted eventually in their disappearance. When these medial changes were of sufficient extent, dilatation and aneurysm formation often resulted. Instances were observed, however, in which the medial damage appeared maximal and yet no aneurysm had resulted. Blood was frequently observed in the damaged media. Since no vasa vasorum were observed, it must be assumed that this blood entered the media through defects in the intima. Extension of blood into this damaged media often resulted in the classical picture of dissecting aneurysm. Severe retrogressive changes without bleeding often resulted in diffuse dilatation, fusiform aneurysm or saccular aneurysm formation. Any of these aneurysms may rupture with fatal hemothorax or hemoperitoneum. In some instances the retrogressive medial changes were repaired by formation of fibers having the staining characteristics of collagen. In no case were new elastic fibers or smooth muscle cells observed in these areas of medial repair. In one animal, fatal hemorrhage resulted from a small focal retrogressive lesion involving all layers of the aortic wall without significant dilatation or aneurysm.

Lesions of the *adventitia* seemed to be secondary rather than primary in that they were not observed in the absence of medial lesions. In cases of aortic dilatation or aneurysm formation without rupture, the adventitia often showed compensatory thickening. This was largely cellular or collagenous, depending on the age of the lesion, but in many cases there was an apparent increase of rather delicate elastic fibers. These elastic fibers did not seem to be derived from the media. Their increased prominence may have been due to the stretching and compression of loose periaortic tissues resulting from the dilatation. Blood was frequently observed infiltrating the adventitia. When the blood was moderate in amount, the adventitia and adjacent periaortic tissues proliferated to form a wall around it. In a few cases quite definite false aneurysmal sacs were formed in this manner. Blood in small amounts in either the media or adventitia was lysed or became organized.

Summary. 1) Weanling, male, albino mice were more resistant to the skeletal effects of diets containing sweet peas or β-aminopropionitrile (BAPN) than were rats. They also showed a much greater individual variation in susceptibility to the skeletal effects of such diets. At sufficiently high levels of BAPN, however, both skeletal and aortic lesions occurred. The microscopic changes occurring in the aorta are described in detail. 2) Paraphymosis also occurred, but the paralysis and urinary retention often seen in rats receiving similar diets were not observed.

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Ploidy Distribution of Nuclei in Rat Liver Following Hypophysectomy.* (23423)

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It has been reported recently(1,2) that following hypophysectomy there is an increase in the ratio of diploid to tetraploid nuclei in rat liver. Bass *et al.*(1) using adult rats observed a 30% increase in percentage of diploid nuclei in the liver. DiStefano and Diermeier(2) have presented evidence that in young rats there is an arrest of nuclear activity following hypophysectomy. This results in maintenance of polyploid distribution present at time of operation. However, neither of these studies took into consideration the effect of reduced dietary intake on the liver. Because severe dietary restriction has been shown to modify polyploid distribution of parenchymal cells(3), it seemed desirable to compare the effects of reduced food intake on this distribution in adult rat liver with the alterations observed in hypophysectomized animals which invariably reduce their caloric intake.

By evaluation of liver samples taken both at time of hypophysectomy and 21 days later at time of sacrifice it was possible to show that the ploidy shifts previously reported(1) were directly correlated with hypophysectomy, as no significant shifts were observed in pair-fed animals of corresponding age and weight.

Methods. 125-135 g Sprague-Dawley rats obtained from Endocrine Laboratories, Madison, Wis., were employed. They were housed individually in a constant temperature room, fed on canned dog food and allowed free access to water. The animals were divided into 3 groups: (1) a group of normal rats fed *ad libitum;* (2), hypophysectomized animals; and (3), normal rats pair-fed with the hypophysectomized animals. All animals were killed by decapitation at the same time each day. A biopsy sample was taken from the tip of median lobe of the liver of each rat in both control groups, at the beginning of the experimental period, and at the time the animals were sacrificed a more representative liver sample was taken. These specimens were fixed in 10% formalin, dehydrated and prepared according to standard histological procedure. Ten percent homogenates of liver tissue were prepared(4) and the nuclei counted on a certified Spencer Bright Line hemocytometer. Mitotic indices were determined on tissue sectioned at 5 μ. The nuclei were stained by the Feulgen technic and the cytoplasm counterstained with fast green. 10,000 nuclei/animal were counted, and values expressed as the number of dividing nuclei/100 parenchymal nuclei. The ploidy distribution of liver nuclei was determined on hepatic tissue sectioned at 20 μ, stained by the Feulgen technic, and DNA content of 50 to 100 whole nuclei estimated by the method of Swift(5).

Results. Data presented in Table I show certain effects of hypophysectomy and reduced food intake on the young adult rat. It is seen that pair-fed animals when compared with hypophysectomized controls have similar changes in body weight, liver weight and nuclei/gram of liver. Therefore, the reduced food intake in the hypophysectomized rat appears to be the major factor responsible for these changes.

Table II shows polyploid distribution of parenchymal nuclei from livers of these animals. Biopsy samples taken at the beginning of experiment have slightly higher diploid values than those taken at the time of sacrifice. This is probably due in part to the location from which the biopsy sample was taken. There are, however, no significant differences between the initial and final samples of hepatic tissue taken from the *ad libitum* control and pair-fed animals. No biopsy samples were taken from the hypophysectomized animals and it was assumed that initial polyploid

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TABLE I. Effects of Hypophysectomy and Reduced Food Intake on Adult Rat.

	Ad lib. control (5)*	Pair-fed control (5)	Hypophysectomized (5)
Body wt, g (initial)	132.3 ± 2.4†	127.3 ± 4.9	123.1 ± 4.5
(final)	273.0 ± 8.9	155.2 ± 10.3‡	138.9 ± 5.4‡
Liver wt, g	12.1 ± .6	5.1 ± .6‡	5.6 ± .4‡
Nuclei $\times 10^8$/g	2.4 ± .2	3.6 ± .2‡	3.9 ± .2‡
" $\times 10^8$/liver	29.1 ± 2.3	17.9 ± 1.0‡	21.5 ± 2.9§

* No. of animals. † Represents a mean figure with its stand. error. ‡ $p < .01$ when compared to *ad lib.* control. § $.05 < p < .1$.

p = probability from Fisher's Table "t."

distribution was the same in the 3 groups. The samples taken from the latter group at termination of the experiment indicate a significantly higher percentage of diploid nuclei associated with a corresponding reduction in tetraploid nuclei. The changes in number of octaploid nuclei are probably not significant, as the number was relatively small in each group. This differs from observations of Bass, Grisham, and McArdle(1), who reported a higher percentage of octaploids in hypophysectomized rats. The reason for this difference is not apparent. However, since synthetic diets were not employed, it is possible that dietary differences could be responsible.

Because it was possible that this increase in percentage of diploid nuclei might be correlated with an increase in percentage of diploid binucleate cells, the percentage of binucleate cells was determined. The results of this experiment are given in Table III. Although a slight but significant increase in percentage of binucleate cells occurred in the livers of hypophysectomized rats, this increase was not sufficient to account for the increased percentage of diploid nuclei shown in Table II. Counts made on stained slides confirmed Harrison's observation(6) that cell suspensions must be employed for this procedure.

To evaluate the possible effect of changes in mitotic activity as an explanation for these ploidy differences between control and hypophysectomized animals, mitotic indices were done on representative samples from each rat liver. These observations are given in Table IV. Mitotic indices were not performed on the biopsied liver specimens because it had been observed in other animals that the percent of dividing cells in such samples was considerably lower than that in more representative samples of liver. Using the biopsy samples taken from the edge of the lobe, a mitotic index of ⅓ to ½ that of a more representative liver sample was obtained. Therefore, mitotic indices were determined on a separate group of animals weighing 133.7 g ± 5.84 g, and an index of .27 ± .025 was obtained. It therefore, may be assumed that the mitotic index in all 3 groups had fallen during the experiments but to no greater extent in one group than another. It cannot be assumed, however, that the rate of fall in each group was the same. The observations of DiStefano and Diermeier(2) would suggest that rate of fall in the hypophysectomized animals is more rapid.

Discussion. The data here presented indicate that the increased percentage of diploid nuclei observed in the liver of the hypophysectomized rat is independent of growth in-

TABLE II. Distribution of Polyploid Nuclei in Parenchymal Cells of Hypophysectomized, Pair-Fed Control and *Ad Libitum* Fed Control Rats.

	Ad lib. control (5)		Pair-fed control (5)		Hypophysectomized (5)
	Biopsy	Final	Biopsy	Final	Final
Class	%				
Diploid	23.3 ± .7*	19.8 ± 2.4	24.0 ± 3.2	19.2 ± 3.2	35.4 ± .97
Tetraploid	72.6 ± 2.6	74.2 ± 1.7	74.0 ± 3.5	77.4 ± 2.7	66.0 ± 1.3
Octaploid	4.0 ± 1.7	6.0 ± 1.6	2.0	3.4 ± .9	2.0 ± 1.0

* Represents a mean figure with its stand. error.

TABLE III. Binucleate Cells in Rat Liver. Comparison of data obtained by 2 methods to show that crowding of nuclei seen in hypophysectomized rats and their pair-fed controls invalidates data obtained from 5 μ tissue sections.

Tissue preparation	*Ad lib.* control (5)	Pair-fed control (5)	Hypophysectomized (5)
		%	
Stained on slides	9.92 ± .7*	17.5 ± .6	16.96 ± .2
Cell suspension	18.90 ± 1.0	20.8 ± .8	23.1 ± .6
		p >.1	p <.01

* Represents a mean figure with its stand. error.

TABLE IV. Mitotic Index Determined on Rat Liver of 5 Rats/Group.

	Ad lib. control	Pair-fed control	Hypophysectomized
	.0	.025	.100
	.018	.020	.010
	.092	.010	.020
	.0	.0	.010
	.020	.0	.010
Mean	.026	.011	.030

hibition of the animal as indicated by normal polyploid distribution observed in pair-fed animals at the end of the 21-day experimental period. Although there is an equivalent reduction in mitotic index in each of the 3 groups of rats during this period, it is probable that this reduction in the mitotic index occurs earlier in the hypophysectomized animal as indicated by the work of DiStefano and Diermeier(2).

It has been reasoned(2) that similar ploidy changes in the liver in young rats are merely a reflection of arrest of liver growth following hypophysectomy. However, the data on older rats reported here cannot be explained in this manner, because the percentage of diploid nuclei is higher in the liver of the hypophysectomized animal than in the control animals of comparable age and weight. Removal of the hypophysis from animals in which the characteristic adult ploidy distribution pattern is established results in a return to a ploidy distribution seen in immature animals. This would suggest that there is a direct relationship between hypophyseal function and the maintenance of normal polyploid distribution of liver nuclei. The particular hypophyseal hormone involved is probably somatotropin as it has been shown that this hormone when given to hypophysectomized rats(2) or mice with congenital absence of the hypophysis(7) will restore the polyploid distribution to normal values. Although the mechanism responsible for this effect is not apparent from the data presented, the data suggest that hypophysectomy creates an imbalance between the rate of DNA synthesis and the rate of mitosis.

Summary. 1. Percentage of diploid nuclei observed in livers of young adult hypophysectomized rats is greater than that seen in control animals of comparable age and weight. 2. In the hypophysectomized rat there is a small but significant increase in percentage of binucleate liver cells. 3. Total reduction in mitotic index in 125-135 g rats is no greater in 21 days hypophysectomized rats than in the unoperated animals; however, rate of change may be more rapid in operated animals.

Technical assistance of Miss Catherine Cheatham is gratefully acknowledged.

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Conditioning of the Mouse Fetus Against X-Irradiation Death. (23424)

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Bisgard *et al.*(1) found that rabbits which had been exposed to x-rays acquired an increased resistance to bacterial toxins. Raper (2) increased the LD/50/30 of mice following total body beta irradiation by giving a prior sub-lethal cutaneous exposure of the same type of irradiation. Cronkite *et al.*(3) increased the radio-tolerance of adult mice by prior exposure to 144 r given at weekly intervals for 3 weeks and followed by a 30-day rest before exposure to 730 r whole body x-irradiation. Thus, a response in terms of radioresistance has been demonstrated to follow low level x-irradiation. It was conjectured that the mouse fetus, while demonstrably radioresponsive, might also acquire some degree of radiation resistance by very low exposure to x-rays during intra-uterine life. The following data are from such a study.

Materials and method. To obtain fetuses of known age, time-matings were made by placing males with non-pregnant females at 5 p. m. and removing them at 9 a. m. the following day. Matings generally occurred toward midnight. Since the youngest fetuses to be x-rayed were 13.5 days it was not necessary to make vaginal smears or to look for vaginal plugs in order to identify mated females. By 13.5 days pregnancies were obvious. Pregnant mice with fetuses ranging from 13.5 to 18.5 days were exposed to x-rays. In the preliminary experiments single exposures of 25 r to 300 r were given to the fetuses. In some cases, there was x-irradiation on 3 or 4 consecutive days of gestation or during early post-parturition so that a higher cumulative dose was received. In later experiments the dose was reduced to 10 r single exposure. The gravid uterus was measured at 50 cm from the target of a Quadrocondex constant potential x-ray tube run at 210 KVP, 15 MA and with 0.28 mm Cu and 0.51 mm Al filtration. The dose rate measured in air but at the level of the gravid uterus was 97.7 r/min. so that 300 r was delivered in 3.1 minutes. At 2 to 4 months of age, fetal-irradiated mice and their parallel unirradiated controls (of the same age) were exposed to doses of x-irradiation ranging from 525 to 625 r. The dose which approximates the LD/50/30 for males is somewhat lower than that for females. For the final experiment a single exposure of 525 r was used, a dose which proved to be 100% lethal for the control males and an LD/50/30 for the females. The lethality data were collected for all mice for a period of 30 days.

Results. Since there were no data for an immunizing type of reaction on the part of the fetus, preliminary experiments included ages from 14.5 days to 3 days post-parturition and the exposure levels from 25 r to 300 r. While these exploratory experiments were instructive, they will be reported here only in summary form. a. Exposure of the mouse fetus to 300 r at 16.5 days was not lethal but increased its radiosensitivity greatly when adult. b. An exposure of 25 r to 100 r gave variable results in tolerance of x-rays when adult, largely deleterious except in those cases where the lower doses were fractionated over several fetal days. (*e.g.* 16.5-19.5). In these latter cases some females appeared to show slight benefit.

The over-all impression from these preliminary data involving some 42 pregnancies and exposures from 25 r to 300 r at different fetal ages was that x-irradiation had a permanent and deleterious effect on the later survival value when the mice were exposed to an LD/50/30 level of x-rays. It was then decided to use a much lower level of single exposure of 10 r given at an earlier fetal age. These data are given in Table I.

The above data indicate that in almost every case there is an appreciable improvement of the tolerance of x-irradiation by mice at 4 months of age by virtue of their exposure to 10 r while *in utero*. Unfortunately, 525 r under the conditions of the experiment proved to be lethal for all control male mice but since

TABLE I. 30-Day Survival % of Mice Exposed at 4 Months of Age to 525 r X-Irradiation, following Fetal Exposure to 10 r.

	Fetal age exposed		30 days, % survival	Increment
♀	Controls (none)		30.4 (23)	—
	13.5 days	+ 10 r all	31.0 (29)	.6%
	14.5		40.6 (32)	10.2
	15.5		54.8 (32)	24.4
	16.5		45.8 (24)	15.4
♂	Controls (none)		0.0 (11)	—
	13.5 days	+ 10 r all	0.9 (29)	.9+
	14.5		14.8 (54)	14.8+
	15.5		17.4 (46)	17.4+
	16.5		9.1 (44)	9.1+

Numbers in parentheses indicate actual No. of mice tested. All are CF_1 white mice.

some experimental males survived in every instance, there is indication of improvement at least to the degree of survival. For the female the LD value was 30% so that the absolute changes could be given.

It is apparent that exposure to 10 r on gestation day 15.5 gave the best results. The male offspring so irradiated showed at least a 17.4% improvement in survival over the controls while the females showed a 24.4% improvement in survival (from 30.4 to 54.8%). The smallest number of experimental animals of either sex used was 24 females exposed to 10 r at 16.5 days so that the data can be considered significant.

Discussion. The causes of x-irradiation death may be numerous and may vary from animal to animal within the same experiment. Some animals die quickly (3-4 days), others in from 10 to 15 days, and some not for 3 or 4 weeks. The causes of death may be quite different at the 3 peaks. In the final experiment reported above most deaths occurred between 8th and 16th days following exposure. There were a few deaths about 24 days, but none during the first 4 days. It is suggested that such radio-resistance as is given to these mice by fetal irradiation may have protected those which would have normally died early within the 30 days following exposure, and a few which might have died late. The cause of death for those dying between 8 and 16 days seems to have been unaltered.

The data reported by Cronkite(3) indicate that most deaths occurred between 9 and 21 days. The resistance provided his adult animals seems to have been somewhat similar to that provided these fetal mice, subsequently exposed to the lethal dose when mature. However, the conditioning of the fetal mice was achieved by a very much lower and single exposure to x-rays.

The mechanism of this increased resistance to x-irradiation death is still completely obscure. It is certainly not due to the selective elimination of susceptible fetuses, for there appeared to be no reduction in litters nor in the normal viability of those exposed to the very low dose of 10 r. It would be extremely difficult to detect any changes in the whole blood, the serum, or in any of the highly radio-sensitive organs such as the spleen, lymph nodes or hematopoietic system following only 10 r exposure. Kiseljov *et al.*(4) recently found that 130 r to adult mice provided increased resistance to ionic emissions, but that subsequent exposures did not have this effect. They attribute this to antibody formation within the mice due to the denaturation of proteins. The data of the current experiment indicate definite improvement of radiation tolerance due to exposure of the fetus, particularly if it occurs on day 15.5.

Conclusions. Irradiation of the mouse fetus at 15.5 days gestation to only 10 r x-rays will give it a considerably better chance of survival if it is subjected to the LD/50/30 range of exposure when adult. Other fetal ages from 14.5 to 16.5 days show some benefit of 10 r exposure, but higher exposures (25 r to 300 r) to any age fetus will have a deleterious effect in later life if the mouse is exposed to x-rays.

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Received June 4, 1957. P.S.E.B.M., 1957, v96.

Determination of Plasma Levels of Tolbutamide (1-Butyl-3-*p*-Tolylsulfonylurea, Orinase*). (23425)

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Tolbutamide (1 - butyl-3-*p*-tolylsulfonylurea, Orinase*) is a potent, orally-active hypoglycemic agent(1). In order to correlate plasma levels of this compound with reduction in blood sugar concentrations, a suitable analytical procedure adaptable to analysis of large numbers of samples is needed. Such a procedure is presented based on the intense absorption by Tolbutamide at 228 mμ.

Materials. A. *Reagents*: (1) M/15 Phosphoric acid: 4.55 ml of concentrated phosphoric acid are diluted to 1000 ml with distilled water. (2) Chloroform: Mallinckrodt A.R. chloroform in sealed glass bottles is used for the extractions. (3) 95% Ethanol: this is redistilled before use. (4) Darco G-60. B. *Apparatus*: (1) Separatory funnels: 60-ml separatory funnels equipped with Teflon stopcock plugs are employed for the extractions. Pledgets of Pyrex wool are inserted into the stems of the funnels and serve to eliminate small droplets of water from the chloroform phase(2). *All* glassware must be free of ultraviolet absorbing substances. Prior to use, the funnels are shaken with 5-10 ml of chloroform which is allowed to filter through the glass wool pledgets in the stems. (2) Filter funnels: 2-inch funnels containing pledgets of Pyrex wool in the stems are used to filter the suspensions of Darco G-60. Prior to use, the funnels and pledgets are rinsed with 95% ethanol and thoroughly dried. (3) Spectrophotometer: a Beckman Model DU Spectrophotometer with 1-cm quartz cells is used for absorbance measurements. Spectra have been obtained with a Cary Model 11 Recording Spectrophotometer. *Procedure.* A. *Extraction*: 1 ml of oxalated plasma is mixed with 1 ml of distilled water and 1 ml of M/15 phosphoric acid in a 60-ml separatory funnel. The acidified plasma is extracted with 10 ml of chloroform by a gentle inversion of the funnel 25-30 times. Occasionally it is necessary to break an emulsion by centrifugation. The chloroform phase is carefully drained through the pledget of glass wool and collected in a vessel suitable for evaporation of the solvent. The extraction is repeated with 2 additional 10-ml portions of chloroform and the extracts combined and concentrated to dryness. In the present study, concentrations have been made in 50-ml round-bottom flasks under reduced pressure below 35°C by means of vacuum evaporating equipment constructed in these laboratories. In other laboratories employing this procedure, evaporation to dryness in a beaker on a steam bath has been satisfactory provided the sample is removed just before going to dryness and remaining chloroform removed by a stream of air at room temperature.† The residue should not be exposed to elevated temperatures but all traces of chloroform must be removed. B. *Charcoal treatment*: the residue is dissolved in exactly 10 ml of 95% ethanol. After sufficient time to insure complete solution, 5-7 mg of Darco G-60 are added and the mixture is swirled several times during 30 minutes. The supernatant solution is then filtered through a 2-inch funnel containing a pledget of Pyrex wool in the stem. C. *Spectrophotometry*: the absorbance of the charcoal filtrate is measured at 228 mμ *vs.* a reagent blank (30 ml of chloroform concentrated to dryness and carried through the remainder of the procedure). A plasma sample obtained from the same individual prior to Tolbutamide administration is also carried through the procedure and similarly measured (plasma blank). The concentration, T, of Tolbutamide (mg/100 ml of plasma) is calculated

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* Orinase is the Upjohn trademark for its brand of 1-butyl-3-*p*-tolylsulfonylurea.

† Marble, A., Joslin Clinic, Boston, Mass., personal communication.

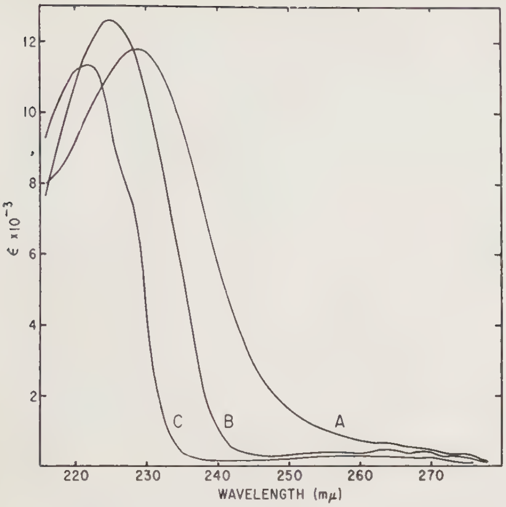

FIG. 1. Absorption spectra in 95% ethanol. A—Tolbutamide (1-butyl-3-*p*-tolylsulfonylurea); B—*p*-Toluenesulfonamide; C—*p*-Toluenesulfonic acid.

from the equation $T = \frac{1000\ A_{228}}{a_{228} \cdot F}$ where A_{228} is the observed absorbance at 228 mμ, a_{228} is the calculated absorbance at 228 mμ for a solution containing 1 mg Tolbutamide/ml of 95% ethanol (about 49), and F is the fraction of added Tolbutamide recovered from plasma by the procedure (about 0.85 for human plasma, 0.91 for dog plasma). This value for plasma Tolbutamide is then corrected for the apparent Tolbutamide concentration obtained for the plasma blank. Both a_{228} and F should be determined for the individual spectrophotometer and laboratory involved.

Results. The absorption spectrum of Tolbutamide in 95% ethanol shows an intense maximum at 228 mμ (Fig. 1). A comparison of this spectrum with those of *p*-toluenesulfonamide and *p*-toluenesulfonic acid (Fig. 1) indicates that the tosyl group is the chromophore responsible for the Tolbutamide absorption at 228 mμ. Absorbance at this wavelength obeys Beer's law over the range 0-24 μg/ml. When Tolbutamide (as its sodium salt) is added to human plasma and carried through the complete procedure the absorbance *vs.* concentration response is linear but is only about 85% of that observed for the pure Tolbutamide standard. A loss of about 15% occurs on extraction and charcoal treatment necessitating use of a correction factor as described above.

An examination of the recovery of Tolbutamide from various activated charcoals has shown recoveries of 92.5% from Darco G-60, 90.0-92.3% from a group of the Nuchars and 74% from Norit A. Since Darco G-60 consistently yields a low plasma blank, it is used routinely. This study indicates that losses of Tolbutamide encountered in this procedure occur, for the most part, during charcoal treatment.

The effect of charcoal treatment on a group of human plasma blanks is shown in Table I. In every case, the apparent Tolbutamide concentration is reduced by Darco G-60 treatment and with the exception of the badly hemolyzed samples, the plasma contribution is equivalent to about 1 mg/100 ml or less.

The recovery of added Tolbutamide from human and dog plasma is shown in Table II. In each case recovery is satisfactory when corrected for inherent losses as described above.

Typical plasma levels of Tolbutamide in the dog following oral administration (as the sodium salt) are presented in Fig. 2. Included for comparison are the corresponding decreases in blood sugar concentrations as determined by the method of Folin and Wu(3). The animals were fasted for 15 hours prior to administration of Tolbutamide and were continued without food during the entire experimental period. Since blood sugar concentrations in fasting controls showed only minor fluctuations, changes due to fasting could be

TABLE I. Effect of Darco G-60 on Human Plasma Blanks.

Plasma condition	Apparent Tolbutamide conc. (mg/100 ml) Before Darco G-60	After Darco G-60
Excellent	2.3	.5
”	1.9	.9
”	1.9	1.1
”	.6	.4
”	1.1	.7
”	1.3	1.0
Slightly hemolyzed	6.9	1.2
Badly ”	11.3	4.0
” ”	10.6	2.3

TABLE II. Recovery of Added Tolbutamide from Human and Dog Plasma.

Human plasma				Dog plasma			
Sample 1		Sample 2		Sample 1		Sample 2	
Added (mg/100 ml)	% recovery	Added (mg/100 ml)	% recovery	Added (mg/100 ml)	% recovery	Added (mg/100 ml)	% recovery
2.3	95.7; 82.6	11.4	101.8; 98.2	9.7	99.0	9.7	99.0
4.6	100.0; 84.8	13.7	99.3; 91.2	19.4	100.5	19.4	99.0
6.9	95.7; 95.7	16.0	104.4; 101.9				
9.1	100.0; 100.0	18.3	105.5; 104.4				
		20.6	100.5				
Mean ± stand. dev. = 97.7 ± 6.4%				Mean ± stand. dev. = 99.4 ± .8			

ignored. It can be seen that maximum plasma levels of Tolbutamide correspond well with maximum blood sugar reductions, and that blood sugar concentrations approach control values as the drug slowly clears from the plasma. Disappearance of Tolbutamide from the plasma is typically first-order. Data in Fig. 2 correspond to half-lives in the dog of 13.7 and 17.6 hours after doses of 25 and 100 mg/kg respectively.

Discussion. Tolbutamide is not a "sulfa" because the *para* position is occupied by a methyl rather than an amino group. For this reason the conventional Bratton-Marshall procedure(4) is not applicable to Tolbutamide and the spectrophotometric procedure described above has been devised.

Sensitivity of the procedure depends on the volume of plasma extracted and the volume of the reconstituted alcoholic solution. The procedure described is useful over the range 1-25 mg of Tolbutamide per 100 ml of plasma. Higher concentrations may be accommodated by suitable dilution of the charcoal filtrate.

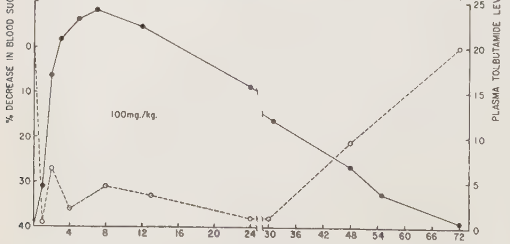

FIG. 2. Relationship of blood sugar decrease to plasma levels of Tolbutamide after a single oral dose to fasting dogs (each point is avg of values obtained from 2 dogs).

Although adequately sensitive, the method lacks specificity. For this reason conditions are presented which result in a minimal apparent Tolbutamide concentration for plasma free of drug (plasma blank). Strong acidification of plasma prior to extraction gives an excellent recovery of Tolbutamide but the plasma blank is higher than obtained on extraction of neutral plasma. Recovery of Tolbutamide from neutral plasma is low due to the fact that Tolbutamide (pKa′ 5.43 at 25°C (5)) exists almost completely in the salt form at the natural pH of plasma. Therefore, some reduction in pH is essential in order to convert Tolbutamide into its chloroform-extractable acid form. Dilute phosphoric acid brings plasma to a pH (4.5-4.8) sufficiently low to give 95-100% extraction but does not increase the plasma blank. Heating the residue following removal of chloroform gives highly inconsistent results. For this reason the dry residue should not be heated but *all* chloroform must be removed as it absorbs strongly at 228 mμ.

Application of the procedure as described, except for the charcoal treatment, to the determination of human plasma blanks has given an apparent Tolbutamide concentration of 2.3 ± 0.9 mg/100 ml. However, an occasional sample has run as high as 15 mg/100 ml. Inclusion of the Darco G-60 treatment (Table I) has consistently lowered the human plasma blank to about 1.0 mg/100 ml or less for clear plasma. Reduction has been even more dramatic in the case of hemolyzed plasma and the occasional extremely high value has been eliminated.

The procedure as described requires no special equipment other than an ultraviolet spectrophotometer. However, the need for scrupulously clean glassware cannot be overemphasized. An analysis may be completed easily within one working day and many may be run simultaneously. The residues following chloroform removal may be stored and this frequently allows a more flexible operation.

After completion of these studies, the method of Spingler and Kaiser(6) appeared, also based on the intense absorption by Tolbutamide at 228 mμ. Utilizing an extraction of lyophilized serum, these investigators compensated for the contribution of the serum by a second absorbance measurement at 280 mμ and treatment of the system as a 2-component mixture (blank + Tolbutamide). Losses of about 30% of the Tolbutamide present were encountered. The procedure presented herein is simpler, losses encountered are considerably less (about 15% for human plasma and 9% for dog plasma), and special apparatus such as lyophilizing equipment is unnecessary. Spingler and Kaiser(6) report a half-life of 8.7 hours for Tolbutamide in the human. The present studies have indicated a half-life of 13.7-17.6 hours in the dog. Species differences in rate of metabolism of the drug appear to be considerable.

Summary. A procedure is presented for determination of Tolbutamide in plasma. The method consists of a chloroform extraction of weakly acidified plasma, concentration of the extract to dryness, dissolution of the residue in a measured volume of ethanol, treatment of the resulting solution with Darco G-60, and measurement of the absorbance of the resulting charcoal filtrate at 228 mμ. Recoveries of Tolbutamide from human and dog plasmas have been satisfactory and plasma levels determined by this procedure have correlated well with the hypoglycemic response elicited by the drug.

The authors are indebted to Dr. Alexander Marble and Mrs. Rachel Leech for making available results of their studies with activated charcoal and to Miss Mildred Prestrud for blood sugar determinations.

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Increased Susceptibility to Herpes Simplex in Mice Subjected to Avoidance-Learning Stress or Restraint.* (23426)

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A variety of factors other than specific immunity modify host response to infectious agents, protecting against repeated exposure to potentially dangerous agents on the one hand, and subtly converting innocuous exposures and inapparent infections into overt disease on the other(1). Psychologic or emotional stress is often proposed as one of the factors predisposing to overt infection(2). Clinical evidence supporting an etiologic role for emotional stress in recurrent herpes simplex is particularly suggestive(3,4), but experimental data are lacking. As the initial step in a study aimed toward elucidation of psychologically induced changes in resistance, an experimental system is described here

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[*] This investigation was supported by research grant from Natl. Inst. of Mental Health, U.S.P.H.S.

which demonstrates a stress-induced increase in susceptibility of mice to infection with the virus of herpes simplex.

Materials and Methods. Four to 6-week old mice weighing 15 to 20 g from a colony of a selected strain of Salmonella resistant Swiss mice were employed.† The mice were fed a commercial mouse ration.‡

Stressing Method. Choice of stressor was guided by 4 considerations: (1) desire for a sustained emotional disturbance, (2) minimization of physical stress which might directly influence resistance, (3) maintenance of nutritional status and (4) applicability to large numbers of animals. Effective, widely employed, stress situations utilizing approach-avoidance conflicts around satisfaction of hunger and thirst were not used because of the second and, particularly, the third considerations mentioned.

The first stressing procedure was an avoidance-learning situation utilizing a shuttle box type of stress cage. Dollard and Miller(5), Mowrer(6) and others have demonstrated that with fear produced by painful shock, animals become rapidly conditioned to the buzzer, light signal and cage in which shock is administered.

The avoidance learning paradigm partially satisfies the requirements listed above for a stress situation. The procedure can be repeated at frequent intervals for several hours. The temporal sequence of stimulus presentations can be varied as disturbance levels decline. At the same time, each mouse, by virtue of avoidance responses, receives shock infrequently. The method does not employ nutritional restriction and is adaptable to large numbers.

Stress cages, 10 x 41 x 5 inches deep, with grid flooring, were partitioned with opaque plastic to form 10 compartments of approximately 4 x 10 inches, each housing one mouse. The outer walls and removable cage tops were of clear plastic to permit observation. The smooth plastic surfaces prevented mice from escaping the stress. Each compartment was divided into two 4 x 5 inch sections by plastic barrier rising 1½ inches above grid floor. A low voltage, low amperage A.C. current could be passed through the grid flooring on either side and through parallel wires ⅛ inch above the top of each barrier to discourage barrier sitting. Presentation, duration, and interval between stimuli were controlled by an electronic programming apparatus permitting independent variation of duration and interval between stimuli. The following sequence was utilized: A loud buzzer and a bright light were turned on for 5 seconds. As this conditioned stimulus, or signal for a shock avoiding barrier jump, was terminated, a current, the unconditioned stimulus, was passed through the grid floor on one side. After 5 minutes, the conditioned stimulus was repeated and the current reversed to the opposite side.

Stress procedure I. At the beginning of each experiment, mice were selected so as to equalize litter representation and mean weight and placed in home cages with food and water *ad libitum*. Each experimental day the animals were transferred from home cages and subjected to the stressing procedure for 6 hours. Control animals were placed in identical cages and were stimulated with light and buzzer, but not current. Food and water were withheld from stressed and control animals during the 6 hour experimental period. Animals in preliminary experiments were observed hourly for avoidance responses and for signs of behavioral disturbance. Cumulative observations of avoidance provided an approximation of amount of shock sustained by animals in subsequent experiments. Behavioral observations on selected animals were utilized in ratings of disturbance levels. At the end of 6 hours, all animals were replaced in home cages.

Within 3 to 6 days, stressed animals learned to avoid the electrical shock 50-80% of the time, and disturbance levels tended to decline. The stress situation was then modified occasionally by changing the duration of the stimuli. The number of days of stress ranged

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† The strain of mice was a highly inbred strain developed by Dr. Leslie T. Webster of Rockefeller Institute for Medical Research. The original pair was obtained from Dr. Howard A. Schneider in 1941 and the colony was maintained by random mating without further selection or inbreeding.

‡ Purina laboratory chow.

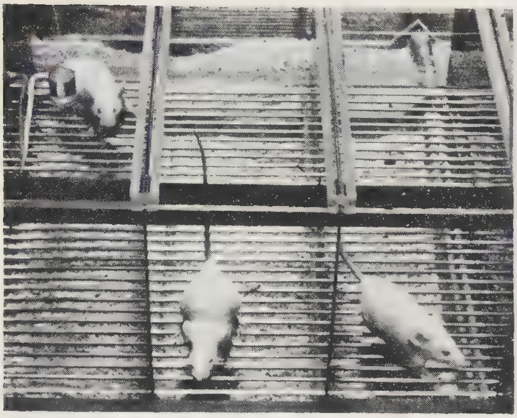

FIG. 1. View of 3 sections of avoidance-learning cage. Photograph was taken a moment after the conditioned stimulus, light and buzzer; the 2 mice on the right have jumped over the barrier and the mouse on the left is preparing to jump to the safe side.

from one to 28 in different experiments. For periods longer than one week, animals were stressed daily for 6 days and rested on Sundays. Stress was terminated at the time of inoculation with virus, and all animals remained in home cages thereafter.

Stress procedure II. The use of the avoidance-learning stress does not completely avoid the possible direct effects of mild electrical shock and increased physical activity. Electrical injury appears unlikely because of the low amperage employed and the activity of stressed animals did not appear to be significantly different from that of controls. However, the 2 possibilities were studied further by use of restraining stress procedure adapted from that employed by Hamilton for rats(7) and by Bartlett, *et. al.*, for mice(8) which eliminated electrical stimulation and minimized physical activity. Experimental animals were loosely wrapped for 6 hours in fine mesh copper screen which produced effective restraint (Fig. 2). Control animals were maintained in home cages from which food and water were removed for the same period.

Experimental infections. The HF strain of the virus of herpes simplex was used. Stocks of virus were prepared in the form of 10% suspensions of pooled infected mouse brain in distilled water and preserved in sealed tubes in a dry ice chest. Animals were inoculated at the desired time by the intraperitoneal injection of 0.1 ml of diluted virus containing 100 LD_{50} as determined by conventional intracerebral inoculation. Virus so administered usually resulted in fatal infection in 50 to 60% of control mice within 14 days. Survivors ordinarily resisted intracerebral challenge, indicating that mice which did not develop overt disease had become immune. The amount of viral antigen in the inoculum was far too small to stimulate significant active artificial immunity; resistance to challenge is, then, evidence of inapparent infection. Animals were observed daily. Variations in the course of the disease in the different groups were manifest as differences in incubation period, in survival time, or in the incidence of mortality. The most apparent differences between experimental and control groups were in survival time and mortality. Survival times and mortality rates were, therefore, employed in evaluating the effect of the experimental procedures.

Results. Avoidance-learning stress. In a representative experiment, Exp. 22, 175 male and female mice averaging 18 g in weight were divided into 4 groups. Group A received avoidance-learning stress 6 hours per day for 28 days. Beginning with the 15th experimental day and continuing for 14 days thereafter, Group B was subjected to the same stress for 6 hours per day. On the 28th experimental day, Group C received 6 hours of stress. Animals in Group D, the control group, were placed in cages of the same type for 6 hours per day throughout the 28 day stress period to equalize the handling factor. On the 29th day, all animals were inoculated with virus. The results of this experiment,

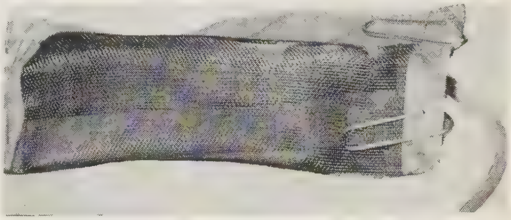

FIG. 2. Mouse restrained in copper screen. Rough edges are covered with masking tape. The front is closed permanently with wire staples; the rear edges are free to introduce and remove the mouse. After the mouse is in place the free edges are overlapped and clamped with paper clips.

TABLE I. Total Deaths and Death Rates in Animals Stressed by Avoidance Learning and Infected with Virus of Herpes Simplex.*

		Daily deaths												Total deaths		
		Days post-inoculation														
Group	N	6	7	8	9	10	11	12	13	14	15	16	Deaths over total N	Groups compared	X^2	Signif. level
A. Pre-inoc. stress 28 day	42	0	6	12	2	3	2	4	2	0	0	0	31/42	A by D A " B	7.73 2.63	.005
B. 14 "	45	2	2	4	6	3	3	3	2	0	0	0	25/45	A " C	8.88	.005
C. 1 "	43	0	2	8	1	2	1	3	1	0	0	0	18/43	B " C B " D	1.65 1.11	
D. Control	45	0	2	3	4	4	1	3	3	0	0	0	20/45	C " D	.06	

* 0.1 ml iper. containing 100 intracer. LD_{50}.

summarized in Table I, suggest a positive relationship between length of the stress and mortality in each group. Chi-square tests for the differences among groups in mortality are recorded in the last 3 columns. Significant differences were found between 28 day stress and control groups, and 28 day and one day stress groups. The number of deaths in the 14 day stress group exceeded that in the control and one day stress groups although the latter differences were not statistically significant for the sample sizes utilized. Groups were also compared for mean and median survival times. An inverse relationship between duration of stress and survival time was suggested, but was not statistically significant.

Confinement stress. No correlation was found in the experiment described in Table I between either mortality or survival period and the amount of shock encountered. In view of the crudeness of the measure, the results could have been due to shock, *per se*, or, possibly, to increased activity. To test these questions, the experiment was repeated, substituting the confinement procedure described, for the avoidance-learning stress. The results summarized in Table II were similar to those obtained with avoidance-learning, although the differences obtained between death rates are smaller, and fewer animals in all groups died. Death rate comparisons again yielded a significant difference between 28 day stress and control animals. No other differences between groups are significant, but the trend of association between mortality and amount of stress is similar to that found in the previous experiment. Tests of survival time did not yield significant differences between groups. However, combined survival time data for the 28 and 14 days stress groups, when compared with similar data for the control and one day stress groups, indicate a trend which is similar to that with avoidance-learning stress. Animals subjected to longer periods of stress tended to succumb sooner than minimally stressed or control animals.

Influence of sex. The experiments presented in Tables I and II suggested a sex difference in resistance to brief, as contrasted to

TABLE II. Total Deaths and Death Rates in Animals Stressed by Confinement and Inoculated with Herpes Simplex.*

		Daily deaths												Total deaths		
		Days post-inoculation														
Group	N	6	7	8	9	10	11	12	13	14	15	16	Deaths over total N	Groups compared	X^2	Signif. level
A. Pre-inoc. stress 28 day	40	0	2	5	3	3	4	4	2	0	0	2	25/40	A by D A " B	3.92 1.21	.05
B. 14 "	37	1	1	7	3	2	1	2	0	1	0	1	19/37	A " C	3.25	
C. 1 "	38	0	2	2	1	2	4	2	1	2	0	0	16/38	B " C B " D	.64 .99	
D. Control	40	1	3	2	2	2	0	3	2	0	0	1	16/40	C " D	.03	

* 0.1 ml iper. containing 100 intracer. LD_{50}.

TABLE III. Total Deaths and Death Rates in Male and Female Mice Stressed by Avoidance-Learning and Inoculated with the Virus of Herpes Simplex.*

		Daily deaths												Total deaths		
		Days post-inoculation														
Group	N	6	7	8	9	10	11	12	13	14	15	16	Deaths over total N	Groups compared	X^2	Signif. level
A. ♀, 14 day pre-inoc. stress	20	0	4	3	3	2	0	4	1	0	0	0	17/20	A by D	5.58	.02
B. ♀, 7 day pre-inoc. stress	20	1	4	4	4	0	0	2	1	0	0	0	16/20	A " B	.173	
C. ♀, exp. cage control	20	0	1	2	1	2	0	2	2	1	0	0	11/20	A " C	4.28	.05
D. ♀, home cage control	20	0	3	2	0	2	1	2	0	0	0	0	10/20	B " C	2.56	
E. ♂, 14 day pre-inoc. stress	19	2	4	1	0	1	1	2	0	2	0	1	14/19	A " E	.765	
F. ♂, home cage control	20	0	1	1	1	1	0	1	1	1	0	0	7/20	D " F	.920	
														E " F	4.91	.05

* 0.1 ml iper. containing 100 intracer. LD_{50}.

longer stress periods. Table III summarizes an experiment designed to test those factors. Eighty females were divided into 4 groups equalized for mean weight and litter representation. As recorded in Table IV, Group A was subjected to 14 days of avoidance learning stress, and Group B to 7 days of stress. Group C animals were placed for 6 hours per day in experiment cages through which no shock was passed, while Group D remained in home cages from which food and water were removed during the 6 hour stress period each day. Fourteen day stress (Group E) and home cage control male groups were also used. Comparison of total deaths in the various groups given in Table III indicates significant differences between females stressed for 14 days and both experimental and home cage controls. More animals died following inoculation in the female group previously stressed for 14 days than in either of the female control groups. A similar significant difference was found between male 14 day stress and control groups. Other differences between conditions, sex comparisons included, did not approach the .05 level of significance. The proportion of females dying in both experimental and control conditions was higher than that found in males. Again, the survival time in both male and female groups subjected to stress tended to be shorter than the survival period for control animals. The difference between the 14 day stress and control groups in the females is somewhat greater than that seen

TABLE IV. Chi Square Test* of Relation between Weight Gain and Death Rates in Experiments Summarized in Tables I, II, and III.

	Within groups			Combined groups		
Exp.	Group	X^2	P level	Group	X^2	P level
E 22	4 wk stress	1.6		4 wk control	9.5	.003
	2 " "	1.2		2 " "	.03	
	1 day "	4.8	.03	4 wk-2 wk	2.9	.09
	Control	1.1		1 day control	1.1	
				ALL GROUPS	8.8	.003
23	4 wk stress	1.06		4 wk control	4.05	.05
	2 " "	.02		4 wk-2 wk	.62	
	1 day "	1.72		2 wk control	1.71	
	Control	.07		1 day control	.51	
				ALL GROUPS	.05	
26	2 wk stress	.50		2 wk control	1.3	
	1 " "	.01		1 " exp. cont.	7.2	.008
	Exp. cont.	.04				
	Cage cont.	.90				

* Fourfold tables, D.F. = 1.

in earlier studies utilizing combined male and female samples.

Influence of stress on growth and of growth on susceptibility. By withholding food and water from both stressed and control animals during the 6 hour stress periods, and providing food and water *ad libitum* for the balance of each day, it was hoped that differences in growth between control and stressed animals would be minimal. However, inspection of the weights of the animals in Exp. 22 and Exp. 23 revealed that the stressed animals did not gain weight at the same rate as did control animals. For example, mean initial weight of the group subjected to avoidance-learning stress for 4 weeks was 20.28 g and mean final weight at the termination of stress was 23.0, a gain of 2.72. Weights of the comparable controls were 20.4 and 25.8 with a gain of 5.4. Similarly, the mean gain for the 4 week confinement stress group in Exp. 23 was 2.17 while the gain for the controls was 5.55.

Was the failure to gain weight directly related to and, possibly, the cause of the observed increase in susceptibility, or was the partial suppression of growth an independent response to the stress situations? Further inspection of growth records revealed considerable variation in response of individual animals. For example, some stressed mice gained at essentially the same rate as did controls while others gained much less rapidly. If increased susceptibility were caused by nutritional deficit and resulting failure to gain weight, a correlation between weight change and susceptibility could be expected within groups of animals subjected to the same experimental conditions. Data from the experiments reported here were examined for this possibility, using chi square to test for association between weight change and death or survivals in each experimental and control condition. The results are summarized in Table IV. As anticipated, the correlation between failure to gain weight at the normal rate and susceptibility is striking when experimental and control groups are combined. On the other hand, of 12 comparisons within groups, only one yielded a chi square significant beyond the .05 level. In most instances the chi squares were negligible and no trend towards positive or negative association was apparent. Thus, when the stress factor was held constant, there was no consistent relationship between susceptibility and weight change and failure to gain rapidly could not be directly related to increased susceptibility.

Discussion. A stress-induced increase in susceptibility to infection with the virus of herpes simplex in mice, manifested by increase in death rates and a trend toward decreased survival times in stressed as compared with control groups was demonstrated. Whether or not our experimental stress is analogous to emotional or psychological stress in man is questionable. However, the stresses resulted in behavioral changes usually associated with emotional disturbance in rodents, *e.g.*, tremor, crouching, defecation, exaggerated startle reaction, and vocalization.

The similarity of response to 2 different forms of stress; the one, fear of electrical shock, in which a possibility of associated electrical injury and increased physical activity could not be completely excluded, and the other, forced restraint, in which direct trauma and physical activity were avoided, suggest a common, centrally mediated response. Additional experimental evidence supporting a relationship between higher nervous centers and susceptibility has been reported by Tobach and Bloch(9) who observed correlations between behavior and susceptibility to tuberculosis in mice and rats and by Teodoru and Schwartzman(10) who have found that a variety of factors influence the susceptibility to poliomyelitis in hamsters. By showing that an environmental situation as routine as that obtaining in daily examination of experimental animals can increase susceptibility to poliovirus, the latter authors emphasize the extreme sensitivity of the balance between host and parasite in their experimental system.

Failure to gain weight as rapidly among stressed groups as among controls, together with the observations of Schaedler and Dubos (11) that mice starved for 24 hours exhibit an increased susceptibility to bacterial infection suggest a direct relation between impaired nutrition and the increased susceptibility observed in our experiments. Conversely, when the response of individual animals was examined, there was no correlation between rate of growth and susceptibility or resistance, a result in accord with experience summarized by Clark, McClung, Pinkerton, Price and Schneider indicating that animals will tolerate severe restrictions of food over long periods of time without significant changes in susceptibility providing specific deficiencies of essential nutrients are excluded(12). In comparing the experiments reviewed by Clark, *et. al.* (12) and the recent work of Schaedler and Dubos(11), the difference in partial deprivation of food or nutrients and complete starvation must be kept in mind. Absence of direct correlation between failure to gain and susceptibility in our experiments indicates that nutritional impairment and increased susceptibility to herpes simplex were independent responses to the experimental stress.

Summary. Stress induced in mice by avoidance-learning or by restraint resulted in an increased susceptibility to infection with the virus of herpes simplex.

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Production of Hypertonic Urine in Humans in the Probable Absence of Antidiuretic Hormone (ADH). (23427)

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Shannon observed the formation of concentrated urines in severely dehydrated dogs with experimental diabetes insipidus(1). Subsequently, Homer Smith and his associates(2) suggested the presence of an "ultimate" concentrating segment (possibly the collecting tubule) where, independently of ADH, water was continuously reabsorbed without solute. In this conceptual scheme, the primary function of ADH is to allow the back diffusion of water when solute is actively reabsorbed in the "penultimate" portion of the distal tubule, permitting delivery of a small volume of isosmotic fluid to the final segment, where a hypertonic urine may be formed.

Recent animal experiments suggested that this does occur and that ADH is not essential for production of a hypertonic urine(3,4). Berliner and Davidson(3) produced "physiologic diabetes insipidus" in dogs by sustained water loading and simultaneously collected urine from each kidney. When the glomerular filtration rate was considerably reduced on one side by compression of the renal artery, urinary flow and solute excretion fell markedly while urinary solute concentration increased to a level greater than that of the plasma. Urine from the contralateral side remained hypotonic, indicating that the experimental procedure did not cause the liberation of ADH. del Greco and de Wardener(4), in somewhat similar experiments, were also able to produce hypertonic urines following depression of the glomerular filtration rate. Finally, Wirz(5) found by micropuncture technics in the mammalian kidney, that during dehydration tubular urine obtained from the distal part of the distal convolutions was isotonic while bladder urine was very hypertonic. This indicated that the actual concentrating process occurred in the most distal segment of the nephron or actually in the collecting tubules.

However, to the authors' knowledge, the effect of acute reductions in filtration rate in humans during maximal water diuresis or in diabetes insipidus has not been described. The present experiments were undertaken to evaluate this problem.

Materials and methods. Two patients, ages 25 and 27, with known diabetes insipidus, served as subjects. In one, the diabetes insipidus was secondary to a head injury (basal skull fracture), and the other was of idiopathic origin. Neither subject showed an antidiuretic response when infused with hypertonic saline; neither was capable of forming a concentrated urine after 10-12 hours of dehydration, and one showed no antidiuretic response to rapid inhalation of 4 cigarettes (nicotine test). Both subjects had random 24-hour urinary volumes of 7 to 10 liters. On the morning of the test, one hour after a breakfast with liberal fluid intake, the subject was catheterized, put in a supine position, and hydrated with 1000 cc of water by mouth. Throughout the experiment a sustained positive water balance of 1000 cc was maintained by intravenous infusion of 2.5% glucose in water in amounts equivalent to his urinary flow. After a constant rate of urinary flow had been obtained in the horizontal position, the patient was elevated to 45 degrees, and the effect of the upright position was observed for 4 to 5, 20-minute periods. At the end of this time, hexamethonium was infused intravenously at a slow rate until a sustained lowering of blood pressure occurred. Usually 5 to 7, 20-minute periods were obtained after initiating the hexamethonium infusion, and the subject was then returned to the horizontal position and urinary collections continued for an additional 6 to 8 periods. Samples of venous blood were drawn approximately 2 minutes before the midpoint of every collection period. Blood pressures were measured at frequent intervals throughout the experiment. The osmolality of plasma and urines was determined by freezing point depression utilizing the Fiske Osmometer. Glomerular filtration rate was estimated from the clearance of inulin. The latter was analyzed in serum and urine by the method of Roe and Epstein(6).

Results. Fig. 1 and 2 illustrate the essential changes observed in both experiments. During initial control periods both subjects demonstrated large volumes of very hypotonic urine (50 and 75 mOs./L., respectively) characteristic of water-loaded and/or diabetes insipidus subjects. On elevation to 45 degrees, filtration rates declined gradually and urinary flows fell progressively. Urinary osmolality increased slightly in both cases (to 80 and 103 mOs./L.). Hexamethonium administration produced marked and sustained reductions in both systolic and diastolic pressures. Almost simultaneously with the sustained hypotension, glomerular filtration rates decreased precipitously to less than 10 cc/minute, and urinary flows decreased to less than 0.5 cc/minute. In both subjects, urinary osmolalities rose promptly and strikingly to hypertonic levels (573 and 763 mOs./L., respectively). As blood pressures began to rise subsequent to termination of the infusion of hexamethonium, and particularly after the patients returned to the reclining position, the glomerular filtration rates rose progressively. In one subject (Fig. 1), urinary osmolality fell rapidly to the previously hypotonic level, while in the other a more gradual return was noted (Fig. 2). Urinary flows increased progressively, but total solute excretions and rates of flow were still substantially below the control reclining periods.

Discussion. It is apparent that in both cases blood pressures were reduced to levels barely consistent with continued glomerular perfusion and filtration. Tubular, pelvic, and ureteral urinary flows obviously were rapidly changing in velocity, therefore errors in rate of

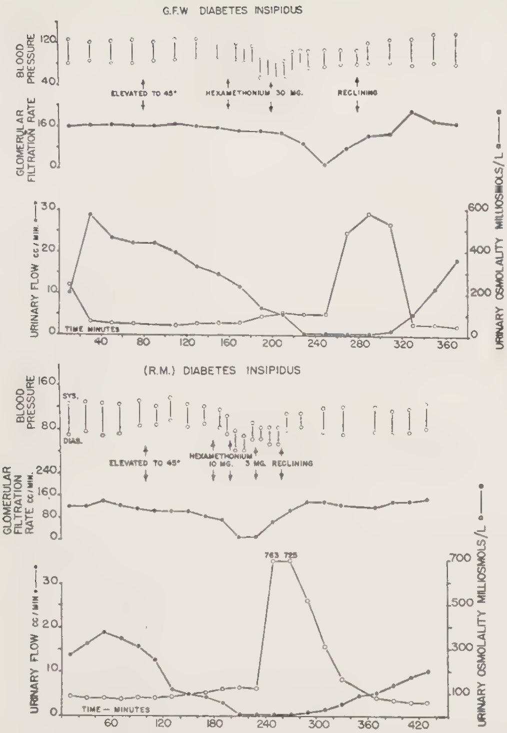

FIG. 1 (top) and 2 (bottom). Effect of acute reduction in blood pressure on filtration rate, urinary flow, and osmolality of patients with diabetes insipidus.

flow and solute concentration due to delay time and residual volumes in the urinary dead space were certainly introduced. These would best explain the slight time lag between the minimal calculated filtration rates and the maximal urinary osmolalities. Similarly, while glomerular filtration was probably reduced to 10 to 15% of normal in both cases, the absolute values shown must be considered only an approximation.

The results of these 2 studies in humans with diabetes insipidus appear to confirm animal experiments of Berliner and Davidson (3) and del Greco and de Wardener(4) that even in the absence of ADH, if the volume of fluid delivered to the concentrating or "ultimate" segment of the renal tubule is small enough, a definitely hypertonic urine can be formed. Although both patients were typical cases of diabetes insipidus, however, it cannot be stated with absolute certainty that the profound reduction in blood pressure did not cause the release of some "residual" ADH in spite of the sustained water load and hypotonicity of the body fluids. This criticism, however, cannot be directed at the experiments of Berliner and Davidson(3), and it seems clear that in the present 2 cases the severe reduction in filtration rate played a fundamental role in the production of the hypertonic urines. Recently a statistical evaluation in humans(7) of the non-hormonal factors affecting characteristics of maximal water diuresis has suggested that glomerular filtration rate varies inversely with the concentration of the urine.

Although reduction in solute excretion was extreme, particularly during the oliguric period, this *per se* is not the cause of the hypertonic urine as suggested by del Greco and de Wardener(4). If glomerular filtration rates are maintained while total solute excretion is reduced by low protein, low salt diets, the normal water-loaded subject excretes a more *dilute*, not a more *concentrated* urine(7).

Summary. The effect of profound reduction of glomerular filtration rate on urinary osmolality of 2 subjects with diabetes insipidus was investigated. In both, reduction in filtration rate was associated with production of a significantly hypertonic urine. These observations support the animal experiments which suggest that ADH is not essential for the production of a hypertonic urine.

The authors wish to thank Miss W. Grant, Miss E. Radler, and Mr. J. Yamashita for their invaluable technical assistance.

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Serologic Response of School Children to Delayed Second Inoculation of Poliomyelitis Vaccine.* (23428)

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In the spring of 1955 scheduled inoculations of poliomyelitis vaccine were greatly deranged by unanticipated shortages of vaccine. Furthermore, many children, having received the first injection in the spring at clinics held in schools, went away for summer vacations and it was not possible or practical even when vaccine was later available to continue mass inoculations until school opened again in the fall. This circumstance necessitated a delay of approximately 5 months between injections and many physicians were concerned about resuming immunization after such a delay. A question frequently encountered was whether or not the entire inoculation series should be started over again. The present paper attempts to answer this question by presenting evidence of the serologic status of children before and after such a delayed second inoculation and comparing the results with those obtained following 2 doses of vaccine administered at an interval of only one month.

Methods. Groups of children in 3 Ann Arbor schools were studied. All were in the 1st, 2nd or 3rd grades of school and none had received vaccine during the field trial of 1954. They had been injected with 1 ml of commercial vaccine in April, 1955, but since a shortage was not anticipated, no blood specimens, unfortunately, had been obtained at that time. Shortly after the opening of school in the fall, clinics were set up in each school to administer second doses of the vaccine 5 months after the first. At this time samples of blood were taken from a total of 221 subjects in 3 schools on the day of the second inoculation and again 2 weeks later. In addition, a small number of children who had not been inoculated in the spring but were receiving vaccine for the first time were studied as controls. Serum specimens were diluted 1/4, 1/8, 1/16, 1/64, 1/256 and 1/1024 and antibody titers against the 3 immunologic types of poliomyelitis virus were determined by neutralization tests against 100 tissue culture doses of virus in stationary tube cultures of HeLa cells.

Results. Table I presents the antibody status of the 221 subjects before and after the delayed second inoculation. It should be noted that these results represent the presence of antibody at any demonstrable titer starting with the lowest dilution tested (¼). It is of considerable interest that so little antibody was demonstrated approximately 5 months following the first inoculation. This may be due in part to a temporal decrease in titer but more likely reflects the inadequacy of the single stimulus. Thirteen percent of the children had no demonstrable antibody to any type of virus prior to the secondary stimulus but none was left without some antibody to at least one type following the second injection. Percentages of children without antibodies to specific types of virus were also drastically reduced following the second stimulus. The most effective antigen had been that of the type II component which left only 23% (13% lacking in all 3 types plus 10% without type II) of the children deficient before the second injection. Only one child (0.4%) however, was without these antibodies after the additional stimulus of the second injection. Although a total of 33% of the children lacked type I antibodies before the second inoculation, only 4% were still deficient afterwards. The type III component of the vaccine was the least effective, leaving altogether 53% without antibodies prior to the second stimulus after which, however, only 12% failed to respond. On the positive side the percentage of subjects having antibodies to all 3 types was increased

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TABLE I. Antibody Pattern of School Children before and after Delayed Second Inoculation of Poliomyelitis Vaccine.

		221 subjects: Delayed 2nd inoculation				10 subjects: 1st inoculation	
		Before		After		Before	After
		No.	(%)	No.	(%)		
No antibody to any type		30	(13)	0	(0)	2	1
No antibody to:							
Type I only	20						
I or II	6						
I or III	19	45	(20)	10	(4)	4	2
II only	4						
II or III	13						
II or I	6	23	(10)	1	(0.4)	5	2
III only	56						
III or I	19						
III or II	13	88	(40)	27	(12)	6	5
Antibody to all three types		73	(33)	189	(85)	3	5

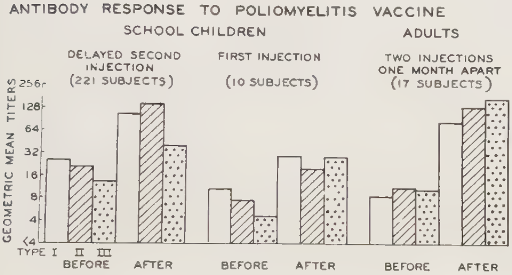

FIG. 1.

from 33 to 85% following the delayed second stimulus. In contrast to these results the antibody pattern of the 10 children receiving their first inoculation at the same time changed very little.

Since the above data were based on the presence or absence of detectable antibody at the lowest dilution tested (¼), the mean increase in actual antibody titer is a better measure of the serologic response of these subjects. Fig. 1 compares the geometric mean titers of children receiving the delayed second injection with those being inoculated for the first time and with a small group of adults which were vaccinated twice at an interval of one month with the same vaccine. The delay did not inhibit a satisfactory response. A better measure of the effectiveness of vaccination is usually seen when only those having no demonstrable antibodies prior to injection are considered. Fig. 2 presents, therefore, the mean titers attained by persons without antibody to any type and by those who had been negative to specific types prior to the second injection. The response was quite satisfactory when compared with that of a small group of adults who were negative to type prior to the first of 2 injections of the same vaccine.

Fig. 3 is a further effort to compare the present results with other groups of individuals receiving only 2 injections of vaccine at an interval of one month. Unfortunately, no data are available in this laboratory on school age children receiving 2 injections 4 weeks apart. However, several groups of infants, pre-school children and adults have been observed as part of other studies(1,2) and are therefore included for comparison. From these data it is apparent that the mean antibody titers to types I and II poliomyelitis are higher in school children following the delayed second inoculation than in any of the

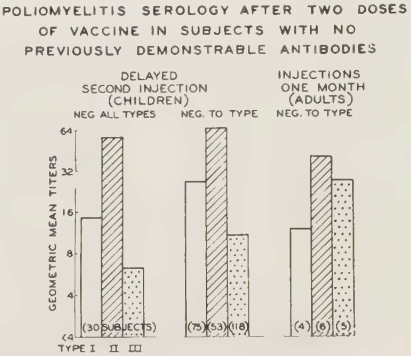

FIG. 2.

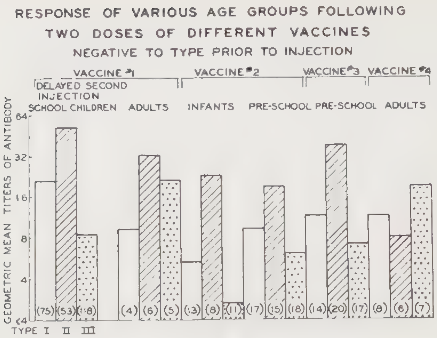

FIG. 3.

other groups which received 2 inoculations 4 weeks apart. Furthermore, only 2 groups, both adults, had higher type III responses than were found in the present study.

Discussion. The data presented are not intended in any way to imply that a deliberate lengthening of the time interval between the first and second inoculation of poliomyelitis vaccine should be practiced, nor are they presented as a substitute for the recommended schedules of inoculation. Salk(3) has recently discussed thoroughly the effect of variations in dosage schedules and has noted that response to multiple injections is enhanced with the increase in time between inoculations. He has pointed out, however, that such an interval would be undesirable if the incompletely vaccinated person by virtue of season or community would be undergoing a greater risk of infection. The present study was made because of the fact that such unforeseen deviations from recommended schedules do occur and raise doubt in the minds of the physicians and of the subjects themselves as to the degree of response under these circumstances.

It is no surprise that a second inoculation delayed 5 months seems to act exactly as a booster injection, the effects of which have been adequately documented for poliomyelitis vaccine(4,5,6). Perhaps the most unexpected finding is the extent of this response when preceded by only a single primary stimulus. Serologically at least the individuals are better conditioned than those receiving 2 inoculations one month apart and not followed up with a third or booster injection.

Summary. The serologic response of school children receiving the second inoculation of poliomyelitis vaccine 5 months after the first injection was studied. Thirteen percent had no demonstrable antibodies to any of the 3 types of virus prior to the second stimulus and many others were lacking in antibodies to one or two types. Only 33% had antibodies to all 3 types. Two weeks following the delayed second injection none was without antibodies to any type, and 85% had antibodies to all 3 types. The antibody levels attained by these children were at least as good if not better than those of other age groups following 2 injections at an interval of one month. The conclusion is made that a delay of 5 months between the first and second injections of poliomyelitis vaccine, although not normally recommended, actually proves advantageous in terms of serologic response.

The author wishes to acknowledge the cooperation and assistance of the following individuals without whose help this study would not have been made: Dr. O. K. Engelke, Health Officer, Washtenaw County Health Department, Dorothy Harding, Director of Nursing Services, Ann Arbor Public Schools, Ethel Hedrick, Eula Avery and Marion Cranmore, Principals of Eberwhite, Mack and Burns Park Schools respectively.

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Studies in Semen Coagulation.* (23429)

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Most mammalian semen coagulates or gelates upon ejaculation. In a few species, such as the dog and the bull, this does not occur. Human semen at first coagulates, then liquefies, presumably as the result of fibrinolytic enzyme action. During the coagulum phase human spermatozoa are hypomotile. As liquefaction proceeds, normal motility appears.

In certain rodents (rat, guinea pig, opossum), ejaculation is immediately followed by formation of a firm coagulum which occludes the vagina. This is termed the "copulation plug" and was described by Lataste(1) who attributed coagulation to mixing of the seminal vesicle fluid with the vaginal secretions. Camus and Gley subsequently observed that the coagulum was formed by the mixing of the seminal vesicle fluid with a secretion which they aspirated from a structure just lateral to the seminal vesicles, and which they thought to be part of the prostate(2,3). These investigators were the first to recognize the enzymic nature of the reaction, and named the "prostatic" component *vesiculase*.

Walker's classical studies have provided the framework for most of the research which has been done since that time in the anatomical and kinetic aspects of rodent semen coagulation(4,5). Walker's principal contributions were identification of the coagulating glands, and of the vital role of their secretion in the clotting reaction, as well as his extensive observations on the chemistry of the clotting process. Various workers(6,7,8,9) have confirmed and extended certain of these observations. Recently Gotterer and his associates (10) have studied the kinetics of semen coagulation in greater detail. The relationship of coagulation to fertility was investigated by Blandau(11) who found that the copulation plug was essential to sperm ingress into the uterine cornua in the rat. In spite of extensive study along this line, however, the exact mechanism of coagulation still remains obscure.

In this report, a simple method will be described for the quantitation of rat seminal coagulation *in vitro*. From our studies, coagulation of normal rat semen appears to take place in 2 separate stages: 1) an initial flocculation, promptly succeeded by 2) gelation and immobilization of the flocculate. By dilution of the components of the reaction, the interval between these 2 stages can be lengthened sufficiently to permit accurate measurements of the coagulation time. Details of the method employed and a preliminary description of attempts to alter the normal rate of coagulation will be considered in separate sections.

Materials and methods. Adult male albino rats were killed by cervical fracture. The coagulating glands were carefully dissected from the seminal vesicles to avoid mixing secretions, and homogenized in 2.0 ml of isotonic saline. This homogenate was diluted 1:10 and 1:50 with saline. The seminal vesicles were excised separately and their secretion collected. Two drops of the expressed secretion were suspended in 1.0 ml of saline. One drop of diluted coagulating gland homogenate (CGH) was placed next to one drop of seminal vesicle fluid suspension (SVFS) on a glass slide, then rapidly mixed with a stirring rod. Floccules appeared almost immediately, and were suspended uniformly throughout the medium. The slide was then slowly tilted from side to side until gelation was complete, as indicated by failure of the floccules to move. The reaction was timed from the point at which mixing of the 2 secretions was begun until complete immobilization of the flocculate had occurred. The endpoint was found to be sharply defined and the elapsed time in seconds was designated as Coagulation Time (CT).

Validity of the method: The coagulation times for rats of the same size have proved reasonably constant and repro-

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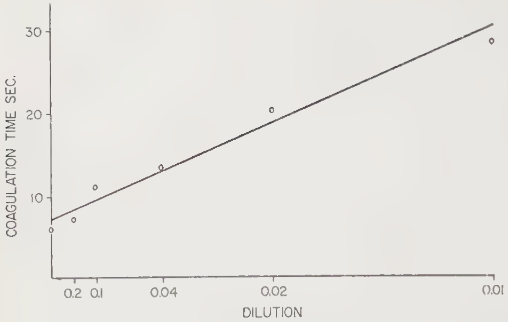

FIG. 1. Effect of dilution of coagulating gland homogenate on coagulation time (CT).

ducible. Five rats ranging between 298 g-320 g were used. Dilutions of CGH of each rat were made to 1:10 and 1:50. CT for each rat at each dilution was determined 5 times against a constant SVFS. Average CT for each rat at both dilutions fell within a narrow range of 8.0-8.8 seconds (1:10) and 20.2-21.4 seconds (1:50). In these studies, pipettes of constant bore were used, providing approximately uniform drop size.

Effect of dilution on CT: Progressive dilutions of CGH in saline were made (1:5, 1:10, 1:25, 1:50, 1:100). Coagulation times were determined using freshly prepared SVFS. The results are plotted in Fig. 1 and demonstrate that CT increases linearly with dilution.

Effect of pH on CT: One drop of CGH (1:5) was added to one drop of SVFS mixed with one drop of 0.067M phosphate buffer and CT determined. The results at different pH levels are recorded in Fig. 2. As shown, the optimum pH of the coagulation reaction is 6.8 to 8.2. This is substantially in agreement with the observation of Gotterer, *et al.* (10) (pH 6.0-8.0) using a turbidimetric method.

Effect of certain dissociated compounds on CT: The effect of certain dissociated compounds as inhibitors of coagulation has been studied. One drop of SVFS (prepared in distilled water instead of saline) was added to one drop of CGH (1:10 in distilled water) mixed with one drop of the test substance (concentration 10^{-3}M). The results are summarized in Table I. It is apparent that Zn^{++} and Cu^{++} prolong the CT, perhaps indicating an inhibiting effect on the reaction. The other substances tested appear to have little or no effect under the conditions of the experiment, except for Na^{+} which appears to shorten the CT slightly. This will be discussed in greater detail below.

TABLE I. Effects of Various Compounds on CT

Test substance	Flocculation	Avg CT (5 determinations), sec.
Control in distilled water	+	10.6
NaCl 10^{-3}M	+	9.9
KCl 10^{-3}M	+	11.1
$MgSO_4$ 10^{-3}M	+	10.9
$ZnSO_4$ 10^{-3}M	+	31.1
$CuSO_4$ 10^{-3}M	+	21.8
NaF 10^{-3}M	+	9.7
NaCN 10^{-3}M	+	11.3

Effect of blood anticoagulants on CT: Table II shows that calcium binding substances, such as sodium oxalate and sodium citrate, have no effect on the coagulation process. Heparin similarly is without effect on coagulation *per se*, but does denature the SVFS, causing flocculation before the addition of the coagulation gland component.

Sodium requirement of vesiculase: In this experiment a crude preparation of the coagulating gland substance (*vesiculase*) was made by treating the clear supernatant of centrifuged CGH with acetone (4°C). The result-

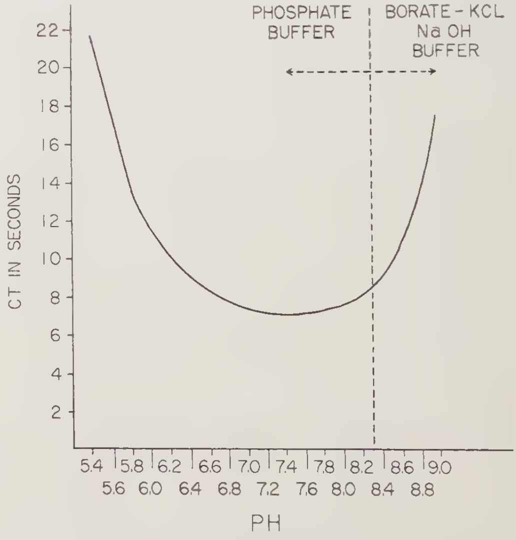

FIG. 2. Effect of pH on coagulation time (CT).

TABLE II. Effect of Blood Anticoagulants on CT.

Substance	Flocculation	Avg CT (5 determinations), sec.
Control in saline	+	8.8
Na Oxalate (100 mg/ml)	+	9.2
Na Citrate (100 ")	+	7.2
Heparin (10 ")	+	7.8

ing acetone powder was suspended in 2.0 ml of distilled water and dialyzed against running tap water for 24 hours. The dialyzate was then tested against SVF in the following diluents: distilled water, NaCl 10^{-3}M, KCl 10^{-3}M and $NaHCO_3$ 10^{-3}M. Table III shows that CT was shortened in the presence of Na^+ ion, indicating that Na^+ seems to be necessary to *vesiculase* action. The Cl^- ion apparently does not play a role in coagulation.

Effect of various physical agents on CT: Most of our experiments confirm the observations of Walker(5) and Camus and Gley(2, 3). It has been found that heating CGH to 100°C, however briefly, destroys its activity. SVFS, however, is not adversely affected by this amount of heat. Cooling of either component to 4°C does not alter the reaction. It has likewise been noted that rapid freezing does not affect coagulating activity. Slow freezing and thawing of the coagulating gland before homogenization, on the other hand, destroys activity.

TABLE III. Determination of Na^+ Requirement of Coagulation Reaction Using Dialyzate of CG Acetone Powder.

Test substance	Avg CT (5 determinations), sec.
SVFS in distilled water + dialyzate of CG acetone powder	18.0
SVFS in NaCl (10^{-3}M) + dialyzate of CG acetone powder	11.8
SVFS in KCl (10^{-3}M) + dialyzate of CG acetone powder	18.8
SVFS in $NaHCO_3$ (10^{-3}M) + dialyzate of CG acetone powder	10.1

Isolation of the coagulating gland factor (vesiculase): Using $(NH_4)_2SO_4$ and acetone precipitation, a fairly potent preparation of *vesiculase* has been obtained from CGH. This has survived unchanged for one year in the refrigerated state. Preliminary fractionation of crude *vesiculase* has yielded a substance which seems solely implicated in the flocculation phase of the reaction.

Summary and conclusions: 1. Coagulation of rat semen seems to involve a diphasic reaction consisting of 1) flocculation and 2) gelation. 2. Velocity of the coagulation reaction can be timed with reproducible results, under the experimental conditions described. 3. The reaction is linear with dilution of the component secretions. 4. Optimum pH range for the reaction lies between 6.8 and 8.2. 5. Zn^{++} and Cu^{++} appear to inhibit the coagulation reaction. Blood clotting inhibitors have no effect. Na^+ seems essential for clotting to occur. 6. Heating of the coagulating gland component destroys activity. 7. Slow freezing of the coagulating gland destroys activity, rapid freezing does not. 8. A crude preparation of the coagulating gland component has been obtained by $(NH_4)_2SO_4$ precipitation. Further work is in progress on purification of this substance.

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Multiplication of Newcastle Disease Virus in Chick Embryo Tissue Culture. (23430)

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Various results have been reported in studies of the quantitative aspects of multiplication of Newcastle disease virus (NDV) in chick embryos and in tissue cultures. For example, the latent period of virus growth in the chick embryo has been reported to be as short as 3 hours or as long as 15 hours(1-3). In tissue cultures of trypsinized chick embryo cells a latent period of 3 to 4 hours was found by one author(4) and a 24-hour period by another, working both with chick embryo and adult chicken lung tissues(5). In view of these discrepancies, it was felt that additional investigations were warranted.

Materials and methods. The glassware used for growth of the tissue cultures was heated in a muffle furnace to 500°C for one hour. The necessity of maintaining separate stocks of glassware for these purposes or the use of special detergents or acids in cleaning was thus obviated. *Virus.* The strain of NDV used was isolated in Israel and designated HP(6). Infected allantoic fluids from the 54-64th passage of the virus in embryonated eggs were used in all experiments here described. *Two media* were used: *M* Hank's balanced salt solution—100 ml, lactalbumin hydrolysate—0.5 g. *M-1* Hank's balanced salt solution 97 ml, lactalbumin hydrolysate—0.5 g, calf serum (from calves aged 6 months to one year)—2 ml. Each solution contained, in addition penicillin (100 units/ml) and streptomycin (50 μg/ml). Medium M-1 was used for growing of tissue cultures. When it was desired to maintain the cells without allowing them to multiply, medium M-1 was replaced by medium M. *Virus titration.* Virus was titrated in the allantoic sac of 9- to 11-day-old embryonated eggs. After infection eggs were incubated 72 hours, deaths were noted and all eggs tested for appearance of hemagglutinins (HA). Only those showing HA activity were considered as having been infected. Titers were calculated in accordance with the method of Reed and Muench(7) and were expressed as log (ELD_{50}) per 0.1 ml of virus suspension. *Preparation of tissue cultures.* Chick embryos were prepared in a manner similar to that described by Dulbecco(8). Nine or 10-day-old embryos were aseptically removed from the eggs and their heads and legs cut off. The embryos were then pressed through a 24-mesh stainless steel wire grid in the bottom of a 50 ml syringe into a vessel containing Earle's balanced salts solution. After 10 minutes (to allow the pulp to settle), the supernatant was sucked off and replaced with 0.2% trypsin in Earle's solution. The suspension was incubated in a water bath at 37°C for 20 minutes, dispersed with the aid of an automatic pipette, and filtered through an Evans filter(9). The filtrate was centrifuged at 3000 rpm for 3 minutes, the supernatant discarded, and the sedimented cells were washed once with Hank's salts solution. The concentration of the final cell suspension was determined in a hemocytometer. M-1 medium was added, to give a final concentration of $1x10^6$ cells per ml, and the suspensions were distributed in 10 ml amounts in sterile, 70 mm Petri dishes. Incubation was carried out at 37°C in an atmosphere containing 4% CO_2.

Results. Tissue cultures, prepared as above, were placed in the incubator. After 48 hours, the supernatant fluids were removed from 3 of the cultures and the cell layer was rinsed with warm phosphate buffered saline. Five ml of a trypsin in Earle's solution was added to each Petri dish, and the dishes were incubated at 37°C for 10 minutes. The cells thus removed from the surface of the glass were concentrated by centrifugation and counted in a hemacytometer. In 3 such experiments the cell count was found to average from 3 to $5x10^6$ cells per plate, and the maximum variations in any one experiment

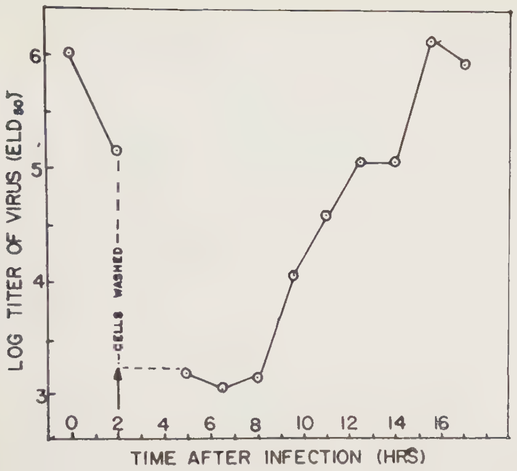

FIG. 1. Multiplication of NDV in tissue culture (chick fibroblasts).

were not larger than 30%.

The supernatant fluids from the remaining plates (usually 5) were removed and replaced with 1 ml of a suspension of NDV in M medium. The amount of virus added was one ELD_{50} to each 10-15 cells in the culture. After a period of time at 37°C to allow for adsorption, the supernatant fluids were removed, the plates were washed repeatedly with fresh medium, and finally 10 ml of medium M was added to each plate. The cultures were returned to the incubator and at various intervals of time, aliquots were taken from each culture, pooled and titrated for infectivity in chick embryos. Control plates showed degeneration of cells only after 4 days of incubation, while in monolayers infected with NDV, destruction of cells was already evident after 3 days of incubation. No attempt was made to correlate the appearance of virus in the medium with the morphological changes in single, virus-releasing cells(11).

The results of 2 such experiments are shown in Fig. 1 and 2. In the experiment represented by Fig. 1, the multiplicity of infection was 0.3, the time allowed for adsorption of virus 2 hours and the average number of cells per plate 3.3×10^6. The virus titer in the supernatant fluids of the cultures remained constant at low level until the sampling performed 9½ hours after infection. This titer probably represented the residual virus remaining after washing of the cultures following infection. The 9½ hour sample showed approximately a 10-fold rise in titer, which continued to rise steadily until 12½ hours after infection. A further rise in titer was detected in the 15½ hour sample.

In the experiment represented by Fig. 2, the multiplicity of infection was 0.07, the time allowed for adsorption 2 hours, and the average number of cells per culture was 3.8×10^6. Samplings were performed at longer intervals than in the former experiment, and were continued until 114 hours after infection. Virus release began 6-16 hours after infection and continued until the 34th hour after inoculation. After that time the virus titer remained more or less constant until the termination of the experiment. The average yield of chick embryo LD_{50} per cell in this experiment was calculated to be approximately 300.

Discussion. The results described above are in good agreement with those of Stulberg, Shapira and Todd(10), who, working with chick embryo fragments embedded in plasma, found that the latent period in the growth cycle of NDV lasted between 9 and 11 hours. However they differ widely from those reported by Levine and Sagik(4), who found that the latent period lasted 3 to 4 hours. Furthermore, the latter authors obtained an average yield of 19-37 virus particles per infected cell, whereas in the experiments described above, the average yield approximated 300 infective particles of NDV per cell in the culture. While it may be that the differences in yield might be due to strain differences in the virus, it seems more likely

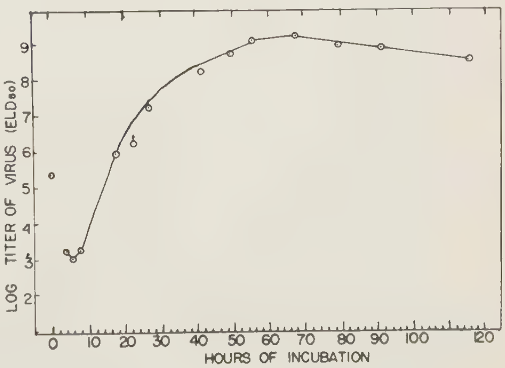

FIG. 2. Growth curve of NDV in tissue culture.

to be indicative of suboptimal conditions in the tissue culture system used in the above mentioned investigations.

It is possible that the reasons for these discrepancies are connected with physiological differences between "attached" and "unattached" cells. Observations made in this laboratory indicate that these differences may be important at least in the case of chick embryo fibroblasts. When cell suspensions were prepared with the aid of 0.25% trypsin, either from chick embryos or from monolayers, the cells failed to attach to the walls of the glass vessels in the absence of serum, retained their globular form, and did not show any obvious metabolic activities such as production of acid in the presence of glucose. In the presence of small amounts of either horse or calf serum (0.1%-0.2%) the same cells attached themselves to the glass walls, took on the typical shape of fibroblasts, and caused acidification of the medium in the presence of glucose, even after removal of the serum by means of washing, and addition of medium from which serum was excluded.

In the system described by Levine and Sagik, although serum was present in the media, other conditions were such that the cells remained in suspensions, *i.e.* in "unattached" form during the period of virus multiplication. It might be that these conditions led to a shortened latent period and lowered virus yield.

Summary and conclusions. The multiplication of cytopathogenic strain of Newcastle disease virus in monolayers of chick embryo cells was studied. The latent period in the growth cycle of this virus was found to last 8-9½ hours, followed by a period of rise lasting 3-4½ hours. The average yield of virus per cell in the tissue culture was found to be approximately 300.

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A Method for Continuous Intravenous Infusion of Dogs Confined to Cages.* (23431)

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The availability of intravenous fat emulsions satisfactory for clinical use has necessitated reevaluation of the efficacy of complete parenteral alimentation. The ideal conditions for investigation of this problem in dogs required that normal animals, unrestrained but confined to metabolic cages, receive intravenous fluids at a constant rate throughout each 24 hour period of study. To fulfill these conditions, it was necessary to devise a method for continuous administration of fluids which would protect the intravenous tubing against mechanical injury resulting from the dog's activity. The swivel developed by Jacobs(1) for the continuous infusion of dogs confined to a room was unsuitable for our work; how-

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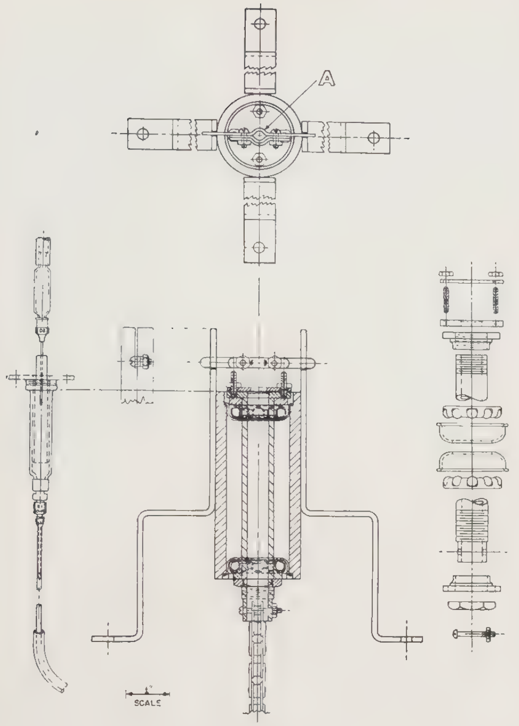

FIG. 1. Individual portions of the swivel assembly drawn to scale.

ever, its modification provided the basis for a simplified swivel which satisfactorily fulfilled the requirements for our studies.

Description of swivel assembly. Fig. 1 represents the individual sections of the swivel assembly drawn to scale. The principle of the assembly is establishment of continuity of the intravenous tubing between the dog and the fluid reservoir by a joint which permits fluid to flow without leakage while the tubing between the joint and the dog is free to rotate without twisting. A 2 cc Leur Lock syringe, its plunger converted into a hollow cylinder by removal of both ends, fulfills the requirements for such a joint. An 18 gauge needle, introduced through a rubber plug which seals the upper end of the plunger, is connected by an adapter to the intravenous tubing from the infusion bottle. A 13 gauge needle attached to the Leur Lock fits snugly into the end of the tubing which connects the dog with the syringe.

The swivel consists of an inner cylinder, free to rotate within an outer cylinder, that is mounted at a convenient distance from the top of the cage. The syringe portion of the assembly, placed within the inner cylinder of the swivel, is supported by the lip of the syringe barrel, which rests upon the top of the inner cylinder. To prevent air from entering the system between the walls of the plunger and the barrel of the syringe, a small vaseline pack is wrapped around the portion of the plunger extending above the barrel. A metal disc is slid over the plunger and bolted to the inner cylinder to hold the vaseline gauze in place and to fix the syringe barrel to the inner cylinder. The plunger of the syringe is immobilized by the screw clamp A (Fig. 1) which is fixed to the stationary outer cylinder of the swivel.

A polyethylene catheter, introduced into the dog's external jugular vein through an incision in the side of the neck, is passed subcutaneously to the back of the neck where it emerges through a small stab wound and makes connection with the intravenous tubing

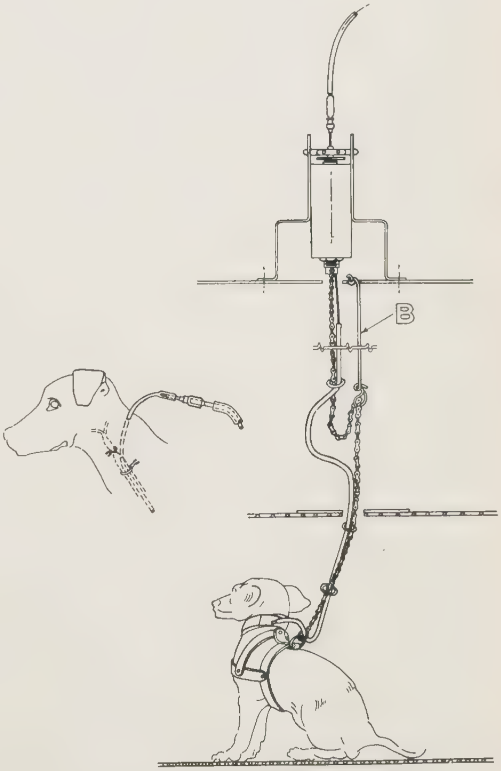

FIG. 2. A schematic representation of the dog's connection to the swivel.

from the swivel. A collar of tape which should not be applied tightly is used to secure the polyethylene catheter and its connection with the intravenous tubing.

The dog is placed in a loose-fitting harness which is connected to the inner cylinder of the swivel by a bicycle chain sufficiently long to allow the animal to lie down but which is shorter than the tubing between the dog and the swivel. Two points on the chain are connected by a piece of rubber tubing (B. Fig. 2) so that the excess chain and tubing are withdrawn from the cage when the dog is standing.

For protection, the intravenous tubing between the swivel and the dog is encased in heavy rubber tubing which is wired at multiple points to the bicycle chain except at the distal end where the excess tubing is allowed to accumulate. Slack in the tubing at this point eliminates the possibility of pull upon the connection between the intravenous tubing and the polyethylene catheter. When the dog lurches, his motion is thus stopped by the chain and no stress is placed upon this union.

Discussion. The swivel has been utilized in studies performed upon 10 dogs. These experiments ranged from 15 to 35 days in duration and were performed without mechanical difficulty. Only one experiment was terminated because the dog chewed the rubber tubing. The careful selection of gentle, quiet dogs will eliminate virtually all the difficulties one may expect with this mechanism. Two solutions were administered simultaneously and, in most experiments, rate of administration for each was controlled individually by glass stopcocks placed in the system between the fluids and swivel. Although this method was satisfactory, it required supervision because of fluctuations in rate of flow resulting from changes in the animal's position. More recent experience has demonstrated that rates of administration can be controlled with greater facility by the use of Sigmamotor infusion pumps.†

The effectiveness of the swivel was singularly dependent upon the bicycle chain. Its flexibility in one plane allowed the dog to stand or lie while its rigidity in all other directions provided an effective handle which rotated the swivel as the dog walked in the cage. Thus the bicycle chain was the link between the swivel and the dog which permitted ambulation without destruction of the intravenous tubing.

Summary. A method for continuous intravenous infusion of dogs confined to metabolic cages has been described. The method was free of mechanical problems and was entirely satisfactory in our experience. It can be recommended for any experiment requiring continuous intravenous infusion of dogs for indefinite periods of time.

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† Sigmamotor, Inc., Middleport, N. Y.

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Role of Cations in Conglutination and in Formation of Properdin Zymposan Complex from Bovine Serum. (23432)

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Bovine serum is an inexpensive and convenient source of properdin(1). However, attempts to study the reaction of bovine properdin with zymosan were complicated by the conglutinating activity of bovine serum(1,2), which causes the clumping reaction known as conglutination. The studies reported here describe the methods developed to prevent conglutination.

Materials and methods. Sheep cells were preserved in Alsever's solution(3) containing penicillin and trisodium ethylenediamine tetraacetic acid(4). Standardized suspensions of sensitized sheep erythrocytes (EA), were prepared as described by Osler *et al.*(5). Veronal buffer(6) containing 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++} was used for dilutions and washing except where noted. All salts of E.D.T.A. were prepared from reagent Na_2H_2E.D.T.A. and adjusted to pH 7.4-7.6 with NaOH. Bovine serum was stored at −70°C in small vials.

Results. Bovine serum was incubated at 37°C for one hour with zymosan,* 5 mg per ml of serum. 0.5 ml aliquots of the absorbed serum, and a control serum incubated without zymosan, were tested for any residual conglutinating activity by addition to 0.5 ml of EA and 1 ml of buffer and incubation at 37°C for 15 minutes. The absorption with zymosan removed the conglutinating activity showed by the control serum.

The role of Ca^{++} in conglutination was studied by adding $MgNa_2$E.D.T.A. to a mixture of EA and bovine serum prior to incubation. The Ca^{++} was bound according to equation (1).

$$MgNa_2E.D.T.A. + Ca^{++} \rightarrow CaNa_2E.D.T.A. + Mg^{++} \quad (1)$$

$SrNa_2$E.D.T.A. and $BaNa_2$E.D.T.A., which chelate Ca^{++} in the same way(7) were tested along with a control tube containing $CaNa_2$ E.D.T.A. It was found that Ca^{++} is necessary for conglutination of EA. Mg^{++} and Ba^{++} are entirely ineffective while Sr^{++} appears to substitute to a limited extent for Ca^{++}. Similar results were obtained with zymosan.

The reversibility of conglutination may be demonstrated by adding $MgNa_2$E.D.T.A. or $BaNa_2$E.D.T.A. to conglutinated EA and incubating at 37°C for a few minutes. The clump disperses and no conglutination can be observed microscopically. After washing these dispersed cells several times with veronal buffer containing no divalent cations, resuspension in veronal containing Ca^{++} restores conglutination.

The reversibility of the reaction suggested that the components of the conglutinating system, except for Ca^{++}, remain bound to the cell during the dissociation phase; or that the components of the system are not bound, but react with, and alter, red cell components so that addition of Ca^{++} causes conglutination. None of the $MgNa_2$E.D.T.A. washings of conglutinated cells showed any conglutinating activity with EA when Ca^{++} was added.

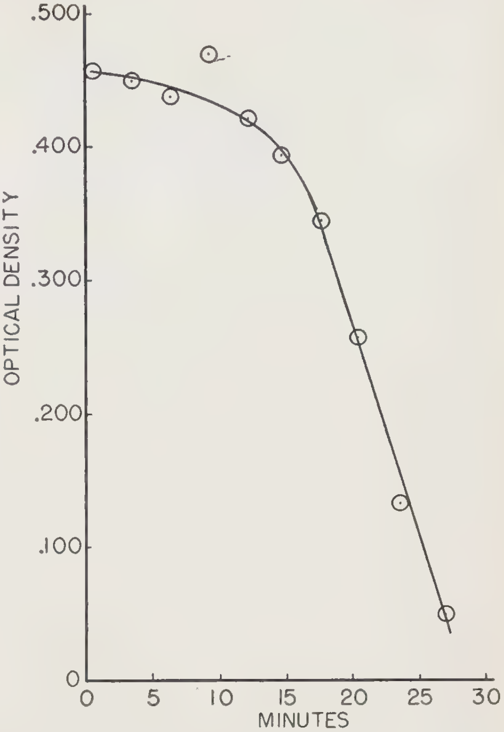

FIG. 1. Inactivation of C′3 by washed bovine PZ formed at 15°C for varying lengths of time. The optical density at 541 mμ measures liberated hemoglobin, and is proportional to C′3 concentration(8).

Since properdin requires Mg^{++}(1) and not Ca^{++} for its reaction with zymosan, the addition of $MgNa_2$E.D.T.A. to bovine serum prevents conglutination of the zymosan, while permitting the zymosan to react with properdin. A kinetic study of the formation of properdin zymosan complex, in the presence of $MgNa_2$E.D.T.A. at 15°C is shown in Fig. 1. The properdin zymosan complexes, obtained at various times, were incubated with C′3 (third component of complement) for one hour at 37°C. The residual C′3 was determined as described previously(8).

After absorption of properdin from serum containing $MgNa_2$E.D.T.A., addition of fresh zymosan and Ca^{++} results in conglutination of the zymosan. Attempts to prepare active conglutinin by dissociation of conglutinated zymosan are in progress.

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* Purchased from Standard Brands, New York City.

Summary. The conglutination of sensitized sheep erythrocytes or zymosan requires Ca^{++}. Mg^{++} and Ba^{++} are inactive but Sr^{++} appears to substitute to a limited extent for Ca^{++}. Zymosan inhibits conglutination of sensitized sheep cells by bovine serum. Bovine properdin and zymosan react readily in the absence of Ca^{++}, but in the presence of Mg^{++}, without any interference due to conglutination.

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A Method of Securing Bacteriologically Sterile Snails (*Australorbis glabratus*).* (23433)

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Recent progress in the axenic cultivation of multicellular organisms suggested that snails might also be cultivated in a bacteriologically-sterile environment. Accordingly, investigations were begun in 1954 to determine whether the eggs of *Australorbis glabratus*, the Western Hemisphere vector of schistosomiasis, could be induced to hatch under sterile conditions after being subjected to surface disinfection.

Materials and methods. 1. *Maintenance and handling of snails and snail eggs.* Eggs were derived from a Puerto Rican strain of *A. glabratus* maintained in our laboratory. The methods employed in rearing these snails have been described(1,2). Since large numbers of eggs were required, several 3-gallon aquaria were set aside for breeding purposes. Each aquarium contained 10 liters of water, a supply of water cress, and 25 snails which were replaced as they died or as fecundity diminished. Water cress was replenished at 5-7 day intervals at which time all egg-masses present on the vegetation were recovered by 'peeling' off the egg-masses using the edge of a watchmaker's forceps. The masses were then kept in a bowl of continuously aerated aquarium water at 76-78°F. Since snail embryos develop readily under these conditions, a supply was always available for experimental use (Fig. 1). Egg-masses containing immature embryos could not be employed since they commonly failed to hatch once disinfected. Consequently, when eggs were required, the stock was examined with a low-power microscope and only 'fully mature' masses selected. Such masses contained embryos which might be expected to hatch within 1-2 days; in fact, a crude guide to a 'mature' egg-mass was provided if one or more snails had already hatched at the time of examination (Fig. 2). The mature unhatched snail nearly fills its egg capsule, moves actively within its confines, and has a fully-formed shell which appears slightly yellow by indirect light. 2. *Composition and preparation of handling solution (HS).* The desirability of using a sterile solution of known composition in which disinfected snails would hatch was apparent. Such a solution was devised empirically by modifying 2 others which had been used, re-

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* This investigation was supported (in part) by a Grant from the Natl. Inst. of Allergy and Infectious Diseases, from the United Fruit Co., and by a Senior Research Fellowship from the Public Health Service.

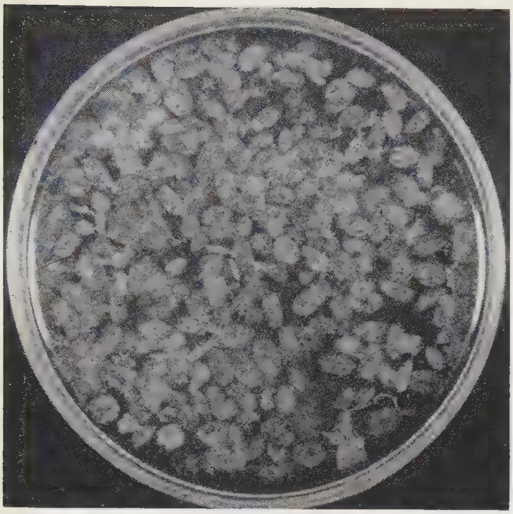

FIG. 1. Stock of *A. glabratus* egg-masses in various stages of development. Shown in an 8 cm Petri dish.

spectively, for snail tissues(3,4) and for physiologic studies on mussels(5). The composition of HS is shown in Table I. Salts #1-4 are dissolved in 800 ml water. Salt #5 is dissolved in 300 ml water and then combined with the first solution. Phenol red is added and the mixture is autoclaved for 10-15 minutes at 10-15 lb pressure. The volume of fluid allows for a 10% loss in autoclaving. The sterile solution is stored at 5°C until needed. The pH of the solution should be 7.2-7.4 after autoclaving; however, after several days the pH drops to about 7.0. If desired, a 4-fold concentrated solution of salts #1-5 may be prepared and later diluted and sterilized. For certain purposes, sterile HS is modified by the addition of: a) $NaHCO_3$ (1.4% aqueous stock sterilized by filtration; use 1.0-2.0 ml stock/100 ml HS); and b) penicillin G (cryst.-pot.) and streptomycin sulfate (100 units and μg respectively per ml HS). HS thus modified is designated HS(M). Glucose (20% aqueous stock; autoclaved) was used only as noted in a few experiments as a further addition to HS(M). 3. *Disinfecting solution.* The use of sodium hypochlorite solutions for disinfecting certain nematode eggs and larvae (see (6) for review) suggested that it might also serve for external disinfection of *A. glabratus* eggs. The solution was prepared by dilution of Clorox,[†] a commercial liquid bleach which contains 5.25% sodium hypochlorite by weight. The physical properties and mode of disinfection of NaOCl have been reviewed(7). 4. *Glassware.* Glassware and rubber stoppers were washed and otherwise processed as for purposes of tissue culture(8). 5. *Sterility tests.* Experiments, except as otherwise indicated, were controlled by inoculation of aliquots of HS(M), snail eggs, or newly-hatched snails into tubes of thioglycollate broth. These controls were maintained at 28-32° C for 7 days and at room temperature for at least 7 days more before being read and discarded. Supplementary bacteriologic tests were utilized

TABLE I. Composition of Handling Solution (HS).

	Constituents	g/liter H_2O†
1.	NaCl*	2.0
2.	KCl	.1
3.	Na_2HPO_4 (anh.)	.05
4.	$MgSO_4 \cdot 7H_2O$	.2
5.	$CaCl_2 \cdot 2H_2O$	.1
6.	Phenol red (0.4% aqueous stock)	.5 ml/100 ml HS

* Merck, sodium chloride for biological work. Other salts reagent grade.

† Double (glass) distilled H_2O used in preparing all salt and stock solutions.

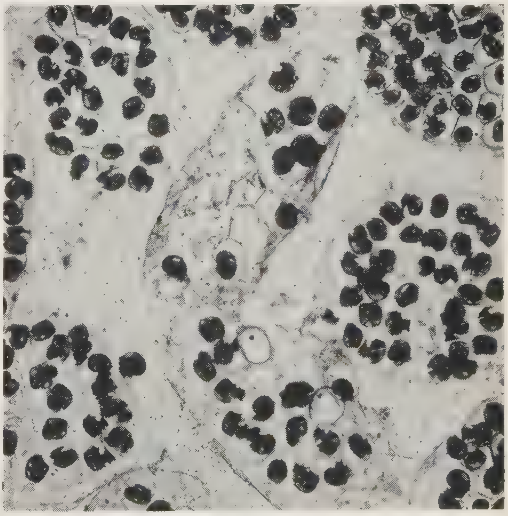

FIG. 2. Well-developed egg-masses of *A. glabratus* (×5). Many snails already hatched from clutches in center.

[†] Made by the Clorox Chemical Co., Oakland, Calif.

when required.

Summary of preliminary experiments. Attempts were first made to surface-disinfect entire egg-masses of *A. glabratus* and permit the snails to hatch under controlled conditions. Groups of mature egg-masses were exposed for 10-15 minutes to Clorox diluted 1:100 with HS(M), washed free of disinfectant, and isolated in separate tubes each containing 5 ml of HS(M). Over 50% of snails in egg-masses so treated hatched within 3-5 days (28°C), but a variable proportion of tubes proved to be contaminated. Further studies revealed that the presence of antibiotics in the HS(M) diluent interfered with the disinfecting activity of the hypochlorite. It was found that the omission of antibiotics during disinfection resulted in an increased proportion of egg-masses rendered sterile, but also in a decrease in rate of hatch; converse results were produced by increasing the concentration of antibiotics present during disinfection. A further complication arose from the frequent detection, by direct microscopic examination of the tubes, of protozoan contaminants. Microscopic examination of mature egg-masses from stock then revealed the occasional presence of ciliates *within* apparently intact, and otherwise normal, eggs. These protozoa superficially resembled forms commonly seen in aquarium water or in egg capsules from which snails had already hatched. The presence of these organisms within snail eggs cannot now be explained, but this observation made it apparent that whole egg-masses could not be used successfully.

Outline of technic. It appeared that the problem might be resolved by disinfecting *individual* eggs of *A. glabratus*. Preliminary experiments to separate eggs from the mass by a variety of enzymatic means were unsuccessful. Attempts were made to free eggs from the mass and from each other by manual means. This ultimately proved feasible and permitted development of the following technic.

1. Stocks of egg-masses are maintained and mature clutches are selected just prior to use. One or 2 mature egg-masses at a time are placed in half a Petri dish containing HS to which 0.2% Triton N-100‡ has been added. The detergent facilitates manipulation since particles do not tend to stick to the dissecting needles. It also rapidly kills bristle worms, protozoa, and perhaps other external contaminants. While snails within intact capsules are not affected by the detergent after 1-2 hours of immersion, those within perforated eggs die quickly. A simple means is thus afforded for distinguishing usable eggs.

2. Dissection of masses is done under a stereo-microscope with 20-30x magnification and transmitted light. Dissecting needles are prepared by breaking the hollow shaft of a 19-22 gauge hypodermic needle from its hub and inserting a very small insect pin ("Minutien", size 0.15) at one end of the cannula; this is crimped firmly into place and the opposite end of the needle is secured in the pin vise of a needle holder. Eggs are separated using 2 such needles. No effort is made to separate the closely adjacent eggs characteristic of most masses; rather, eggs surrounding one to be isolated are punctured and the selected egg freed by a 'scissoring' action of the 2 needles. However, in some clutches eggs do not lie immediately adjacent to one another and it is possible to separate them rapidly. Attached debris is trimmed away as each egg is separated from the mass and the eggs are then transferred by pipette to a dish containing HS. When the desired number of eggs has been accumulated, they are washed several times with HS (by decantation) and are checked microscopically for viability. A normal snail may be recognized by gross movement and by its characteristically rapid heartbeat. Eggs in which the snail is withdrawn, in which the capsule appears to be collapsed or punctured, or in which protozoa are observed, are discarded. The process of securing individual eggs becomes rapid with practice. An experienced person can free 50-100 viable eggs (Fig. 3) in about an hour, the variation depending on the nature of available masses.

3. The remaining procedures are done macroscopically, preferably in a hood, with sterile equipment and precautions. The eggs are transferred in groups of 10-20 to screw-cap

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‡ Made by Rohm and Haas, Philadelphia. Other detergents were not investigated.

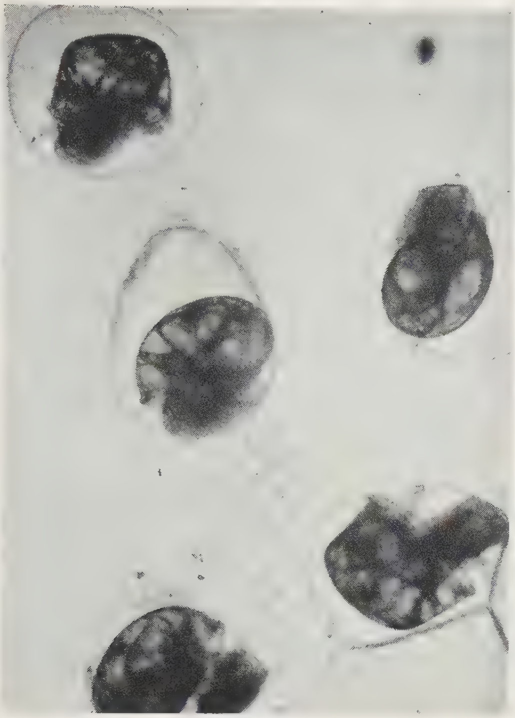

FIG. 3. Characteristic appearance of separated eggs of *A. glabratus* containing fully-developed snail embryos (×28).

vials (20x75 mm) using a Pasteur pipette. A minimum of fluid should be carried over, and fluid and eggs should be deposited on the bottom of the vial without contaminating its sides. All but a small amount of the transfer fluid is removed using the same pipette, and the cap is tightened to prevent drying.

4. Disinfecting fluid is prepared as follows: Place 19.95 ml HS (room temperature) in a sterile container. Add 0.05 ml (approximately one drop) of Clorox using a 1.0 ml serological pipette; this represents a 1:400 dilution of Clorox stock. Upon addition of Clorox the HS will turn deep purple, and it should be used within a few minutes.

5. Transfer 5 ml of the disinfecting solution to a vial and flood the eggs with it, tapping gently to swirl them about. Agitate the contents once or twice during the next 10 minutes. After 10 minutes the solution is removed by aspiration, using a narrow-bore Pasteur pipette. The eggs are washed free of the disinfectant with three 5 ml changes of HS(M), the wash fluids being removed by aspiration. About 2 ml of the last wash are left as transfer fluid.

6. A separate Pasteur pipette is used for the contents of each vial. Eggs are taken up in small groups and transferred to previously prepared tubes (*i.e.* 20x150 mm culture tubes containing 5 ml, or Wasserman tubes (10x100 mm) containing 2 ml of HS(M)), and one egg is deposited per tube; these are closed with rubber stoppers or screw caps. After completion of the procedure tubes are examined (using a stereo-microscope) in the same manner as roller tube tissue cultures. Snails which may accidentally have been killed during treatment are discarded. Tubes are maintained in an upright position in an incubator at 28°C.

Summary of results. 1. The concentration of disinfectant and the treatment period indicated above were selected after experiments in which the 2 factors were varied. In each such experiment, snail eggs were disinfected in groups of about 10 (using one of the schedules shown in Table II), washed and isolated in tubes containing 2 ml HS(M). The tubes were examined daily and hatch records compiled. On the 7th day each snail (or unhatched egg) was transferred by Pasteur pipette to a tube of thioglycollate for sterility tests. It is apparent from the summarized data (Table II) that there is some freedom in the choice of treatment schedule, and that the selection of Clorox 1:400 for 10 minutes seemed to strike a good balance between % hatch and % rendered sterile. Further experience with this schedule has shown that hatch and sterility rates of 90-100% can usually be obtained.

2. A subsequent experiment was aimed at more rigorous bacteriologic screening for contaminants. Four groups of mature eggs (totalling 56) were disinfected and tubed, and during the succeeding 7 days 44 (79%) hatched. On day 7 each snail or unhatched egg, together with some HS(M) from its tube, was transferred to thioglycollate medium. Aliquots of the remaining HS(M) from each tube were inoculated into nutrient broth, a blood agar slant, and a slant of Sabouraud's dextrose agar. Tubes containing solid media

TABLE II. Effects of Time and Concentration of Clorox Disinfectant on Hatch and Sterility Rates of Eggs of *Australorbis glabratus*.

			Separated eggs of *A. glabratus*				
						Sterile snails or eggs†	
Clorox in HS	Min. treatment	No. exp.	No. treated	No. hatched*	% hatched	No.	%
1:400	2	6	85	52	61	74	87
”	5	7	77	63	82	63	82
”	10	5	50	39	78	47	94
”	15	4	37	25	68	37	100
1:200	2	5	45	29	64	44	98
”	5	5	48	28	58	47	98
”	8	3	28	15	54	28	100
1:100	2	3	30	15	50	30	100
Totals		38	400	266	67	370	93

* By day 7 after treatment.

† ‘‘Eggs’’ refers to those which did not hatch by day 7.

were sealed with Parafilm to prevent drying during the incubation period. All tests were held at 28-32° C for 10 days and then at room temperature for an additional 21 days. No contaminants were detected in this series.

3. Experiments were done to determine ability to hatch and length of survival under different sets of sterile conditions, eggs having first been disinfected in groups and tubed individually using the routine procedure. Maintenance conditions were as follows (all at 28° C): a) In cotton-stoppered Wasserman tubes containing 2 ml HS(M), (9 experiments). The initial pH was 7.6-7.8 but rose rapidly to 8.5-8.7 or higher. In no case was the original HS(M) replenished or changed, although considerable loss through evaporation occurred during the observation period of several weeks. b) As in ‘a’ but with 500 mg% glucose added (6 experiments). c) As in ‘a’ but tubes closed with rubber stoppers (3 experiments). The initial pH was 7.6-7.8 and no changes in pH were observed during several weeks of observation. d) As in ‘c’ above but bicarbonate omitted (3 experiments). The initial pH of 6.8-7.0 remained during the period of observation. In these experiments a total of 340 mature eggs were treated and tubed, 292 (86%) hatched, and only 9 (3%) of the hatched snails or unhatched eggs proved to be contaminated. Variations in hatch or sterility rates among the several experiments were negligible. All tubes were examined daily and a snail was recorded as having died only when heartbeat could not be detected. Using 50% survival, in terms of days after hatch, as an index, there were no significant variations in length of survival, although snails in HS(M) plus glucose tended to live slightly longer. Thus, all the 50% end-points fell between 11 and 20 days with the majority of them falling between 15 and 20 days. Deaths were rare in the first 5-day period after hatch, a few deaths occurred in the next 5-day interval, with a sharp increase during the succeeding 10 days. Maximum survival in the various maintenance conditions ranged from 21-29 days except in one instance (in group ‘d’) in which a single snail survived for 52 days. Similar observations were made when treated eggs were isolated in 5 ml HS(M) (500 mg% glucose in one group) in rubber-stoppered 20x150 mm culture tubes. The results paralleled experiments noted above in which smaller fluid volumes in smaller tubes were used.

4. The heartbeat rate of newly-hatched (less than 24-hour old) *A. glabratus* maintained unfed in HS(M) at 28°C under sterile conditions usually averaged 100-120 per minute. This rate declined steadily during the ensuing 5 days to about 60-80 beats per minute. No effects on rate of heartbeat were observed due to the presence of bicarbonate or glucose during this 5-day interval. Similarly, no differences were noted in comparing snails kept in cotton-stoppered tubes or in tubes sealed with rubber stoppers. However, temperature did affect rate of heartbeat. Three groups of 20 eggs each were disinfected and

the eggs isolated in 2 ml of HS(M) (plus 500 mg% glucose) in rubber-stoppered Wasserman tubes maintained at 24, 28, and 32°C, respectively. The proportion of hatch was high in all 3 groups (18-19/20). However, on day 5 (calculated from day of hatch for individual snails) the mean rate of heartbeat for the group at 24° was 73 per minute, at 28°, 82 per minute, and at 32°, 97 per minute. Rate of heartbeat beyond the 5th day post-hatch varied greatly, presumably reflecting individual differences in response to inanition.

5. A variable degree of success was achieved in attempts to secure viable, bacteriologically-sterile snails other than *A. glabratus*. The outlined procedures were applied to mature eggs of *Biomphalaria pfeifferi* and results comparable to those with *A. glabratus* were obtained in regard to rates of hatch, sterility, etc. However, preliminary experiments with mature eggs of *Lymnaea* sp. and of *Marisa cornuarietis* were not similarly successful.

Discussion. Some interesting aspects of the biology of *A. glabratus* emerge from the foregoing studies. It is apparent that microbial action is not essential to the hatching process, that snails will hatch in a simple solution of salts very different from the complex composition of pond or aquarium water, and, that once hatched, they may live for long periods of time without food. The behavior of the newly-hatched isolated snail appears unaffected, except as manifestations of inanition develop, by the absence of living micro-organisms, by differences in pH in the range indicated, by the presence of antibiotics or phenol red, or by being confined to small volumes of fluid in sealed tubes.

The behavior of the normal snail *in vitro* before eclosion is rather characteristic. The snail's foot is almost always maintained in contact with the inner wall of the egg capsule even when the snail is not moving about actively. Heartbeat is distinct. Although the cilia coating the tentacles and other exposed surfaces cannot be seen under the low-power microscope, the currents they produce are evidenced by movement of the particulate matter usually present in the fluid of the egg. The egg capsule itself is slightly yellow in color and usually retains its firm-looking, elliptical outline until the process of eclosion begins. This is initiated by a 'stretching' action, in which the snail appears to brace its shell against one side of the egg capsule and extend its foot against the opposite side, thus tending to distort the outlines of the egg. As the snail moves along the inner side of the egg repeating this movement, the egg capsule eventually takes on a crinkled or partially-collapsed appearance. The snail's mouth parts are in frequent motion during this process, and eventually a small rent appears in the capsule through which the snail ultimately escapes. The snail may thereafter be seen crawling on its empty egg capsule but no effort to eat it has been observed.

Once free of the egg capsule, the newly-hatched snail (which averages 0.7 mm in diameter) moves about actively with the foot in contact with the wall of the culture tube. However, after about 10 days some snails may be found detached from the walls. The foot, while partly withdrawn, may still be observed to move. Heartbeat, although slow and irregular, may be observed for several days to several weeks after other objective signs of life have ceased. By the time heartbeat has nearly stopped, the animal's soft parts appear translucent.

The accumulated experience of rendering several thousand eggs bacteriologically sterile indicates that 90% or more hatch during the first 3-4 days after treatment and only a negligible proportion thereafter. Nothing has been noted to explain why some apparently normal mature eggs fail to hatch, even though many have been observed to live for as long as 2 weeks. In a different category are eggs which contain immature embryos at time of treatment, since a very high proportion of such eggs regularly fail to hatch. There are at least 2 other circumstances in which rate of hatch may be materially reduced: a) If mature eggs are subjected to too high a concentration of disinfectant, or if they are exposed for too long a period of time, the unhatched snails usually die within 1-2 days. The survivors rarely hatch; the foot is usually retracted and may appear partially disintegrated and bordered by loose cellular elements. b) Hatching is also inhibited by the antifungal antibiotic Mycostatin§ at certain concentrations. This substance was added to a few tubes in some of the preliminary experiments in an effort to suppress mold contaminants. In concentrations of 12.5 or 25 units per ml molds were suppressed but few or no snails hatched; at 5 units per ml a small proportion of snails hatched but molds were not uniformly suppressed. It is of interest that a very low concentration of Mycostatin is rapidly lethal to adult *A. glabratus*(9).

The development of a technic for securing newly-hatched, bacteriologically sterile, *A. glabratus* should permit a variety of additional studies. For example, the metabolic characteristics and nutritional requirements of the young snails can be investigated in the absence of micro-organisms or in the presence only of selected micro-organisms. Furthermore, snail tissues free of contamination can now be secured for *in vitro* cultivation. Finally, 'germ-free' snails maintained under sterile conditions should provide an ideal tool for screening and studying microbial or viral agents which might be of value in biological control. Aspects of these problems are now under investigation.

Summary. Details of a technic are presented by means of which newly-hatched, bacteriologically sterile snails (*Australorbis glabratus*) can be secured for study. Snail eggs containing mature embryos are separated manually from the egg-mass, disinfected externally with dilute sodium hypochlorite, and isolated in sealed tubes containing a suitable salt solution and antibiotics. Using this procedure, 90-100% hatch occurs within 3-4 days of treatment, and tests have shown that only a negligible proportion are bacteriologically contaminated. Details of maintenance, behavior, and longevity of these snails are discussed.

I am indebted to Dr. E. H. Michelson for maintaining the colony of *A. glabratus* from which the eggs used in these studies were derived; to Dr. John H. Hanks, Harvard Medical School, for suggestions regarding the composition of HS; and to Miss Sarah E. Dwight for technical assistance.

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§ Squibb brand of Nystatin. Kindly supplied by E. R. Squibb and Sons, New Brunswick, N. J.

Observations on *in vitro* Behavior of Altered Mononuclear Phagocytes Following Exposure to Tubercle Bacilli.* (23434)

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Although cellular response to infection with the tubercle bacillus has been extensively investigated, there is considerable difference of opinion as to the role of phagocytes in tubercular infections(1,2,3). Mononuclear phagocytes have been proposed as the main factors of resistance to tuberculosis infection(4,5). Vorwald and co-workers(6) indicate that BCG will produce fatal cavitary disease in silicotic guinea pigs. Dubos(3) pointed out that disease in the silicotic individual infected with BCG develops because silicosis changes the physicochemical environment of the tissues. These changes allow bacillary growth in excess of that obtained in normal tissues. The effect of various forms of silica on motility and metabolism of phagocytes has been described by Vorwald and Delahant(7), and Gardner(8). However, adequate descriptions of the incidence of phagocytosis of tubercle bacilli by cells "blocked" or altered through ingestion of various particles, including silica, are not available. In the present paper, observations of the behavior of "blocked" or altered mononuclear phagocytes toward tubercular infections by virulent and attenuated strains are described. "Blockade" of the monocytes was accomplished with trypan blue, silicia, carbon, coal dust and cement particles.

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* This investigation was supported in part by grant from Public Health Service.

Materials and methods. Female albino guinea pigs, weighing between 300 and 400 g were used. All animals were tuberculin negative. A chemotactic agent, 10 ml of a 1% starch solution, was injected by the intraperitoneal route into each animal. "Blockade" of the mononuclear phagocytes was accomplished by intraperitoneal injections of 10 ml of a 0.5% solution of trypan blue, 8% solution India ink (Higgins liquid solution), 5% silica suspension, 1% coal dust suspension (Bituminous-West Kentucky Vein) or a 2% suspension of Portland cement on the 4th and 5th day after injection of the starch solution. The blocking agents were injected into groups of at least 20 animals with equal numbers receiving injections of normal saline. Trypan blue and India ink were colloidal solutions while silica, coal dust and cement suspensions contained particles of less than 43 μ size as determined by a standard sieve.

Exudate was collected from all experimental animals on the 6th day according to the method described by Suter(5). Suspensions of the strains of tubercle bacilli[†] used, were obtained by differential centrifugation of 8-10-day-old Tween-albumin cultures. Bacterial numbers were estimated turbidimetrically through the use of a Bausch and Lomb spectrophotometer. The bacterial suspension was diluted with Tween-albumin medium so that 5 ml contained a final concentration of approximately 2 x 10^6 viable units of tubercle bacilli.

After addition of fresh homologous serum in a final concentration of 2.5% to the exudate, 8 ml of the exudate was mixed with 2 ml of the standardized bacterial suspension. The experimental series consisted of "blocked" and "non-blocked" monocytes plus H37Rv or BCG. Other cultures of monocytes were also maintained in the absence of tubercle bacilli. The mixtures were pipetted in 0.5 ml amounts onto ⅞ inch square coverslips in sealed partitioned Petri dishes and incubated at 37°C. Coverslip cultures were selected at 1, 2, 4, 8, 16 and 24-hour intervals and stained by the Ziehl-Neelson method. A phagocytic index was obtained by determining the percentage of mononuclear phagocytes containing tubercle bacilli in each of 200 cultures. In all instances, a minimum of 100 cells in 2 or more diameters of a coverslip culture were counted.

Results. Comparison of the phagocytosis of *Mycobacterium tuberculosis* strain H37Rv by unaltered and altered mononuclear phagocytes of guinea pig exudate is presented in Fig. 1. Although observations were made at 8, 16, and 24 hours, no significant difference in degree of phagocytosis was noted. For this reason no results beyond 4 hours are reported. These results show that all agents tested significantly inhibited phagocytosis of the test organism. Minimal phagocytosis was observed in the presence of carbon particles. Although phagocytosis in the presence of trypan blue was greater than that observed with other "blocking agents," it was significantly lower than that observed in the "non-blocked" or unaltered cells.

Observations on the phagocytosis of *Mycobacterium tuberculosis* strain BCG by altered and unaltered monocytes are presented in Fig. 2. Although phagocytosis was decreased in the presence of all agents tested, it was greater than that observed with the same

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[*] H37Rv was obtained from the Trudeau Laboratory, Saranac Lake, N. Y., BCG was obtained from the Henry Phipps Institute, Philadelphia, Pa.

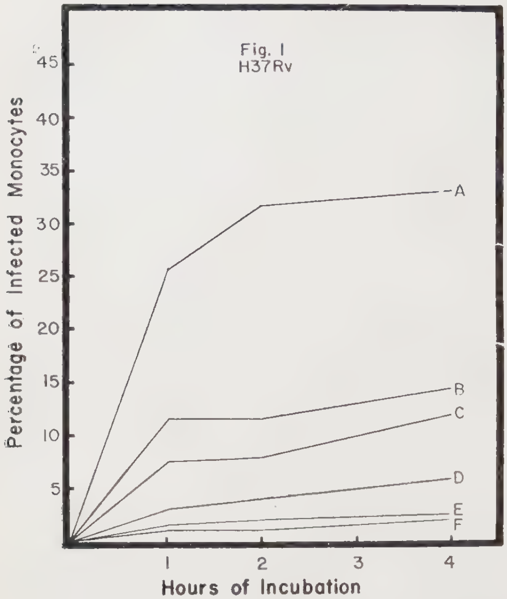

FIG. 1. Phagocytosis of H37Rv by altered and unaltered mononuclear phagocytes. A—Monocytes with no altering agent; B—monocytes with trypan blue; C—monocytes with cement; D—monocytes with silica; E—monocytes with coal dust; F—monocytes with India ink.

agents in presence of the virulent organism. The pattern of relative effectiveness of "blocking agents" observed in the experiment with the virulent organism was apparent in this experiment with the BCG strain.

Discussion. Although the phagocytic cells were subjected to 2 days of *in vivo* exposure to the various altering agents, the ingestion of particles varied. It is recognized that the starch solution, which was used as a chemotactic agent, might have contributed to some extent to the "blockade" of the phagocytes, as well as stimulating new cell activity. This additional effect of starch solution does not appreciably alter interpretation of results since the same quantity was used to produce all the peritoneal exudates and all cells were counted. Some of the altering agents like cement and silica may cause toxic effects at high concentrations which may affect new cell activity or blockade(9). However, in the concentrations used here, percentage uptake did not vary significantly from uptake when relatively non-toxic particles like carbon particles in water (India ink) were used for altering. Prior to 24 hours there was no evidence of intracellular multiplication as Mackaness pointed out(1). Therefore, it is felt that these responses represent phagocytosis without subsequent multiplication.

Our observations indicate that ingestion of various particles significantly decreased the ability of mononuclear cells to phagocytize both attenuated and virulent strains of the tubercle bacillus. There was a higher average uptake of BCG than H37Rv by both altered and unaltered monocytes which may be due to the tendency of attenuated forms to be more readily phagocytized than their virulent forms. Other investigators(6,8,10) have shown that phagocytic ingestion of dust particles resulted in altered activities of these cells, but the effect on ability to phagocytize has not been described. Kato and Gozsy(11) have recently demonstrated that low concentrations of histamine and varying concentrations of antihistaminics inhibit phagocytosis. The mechanism of these observations does not appear readily apparent, but is not likely related to the results described here.

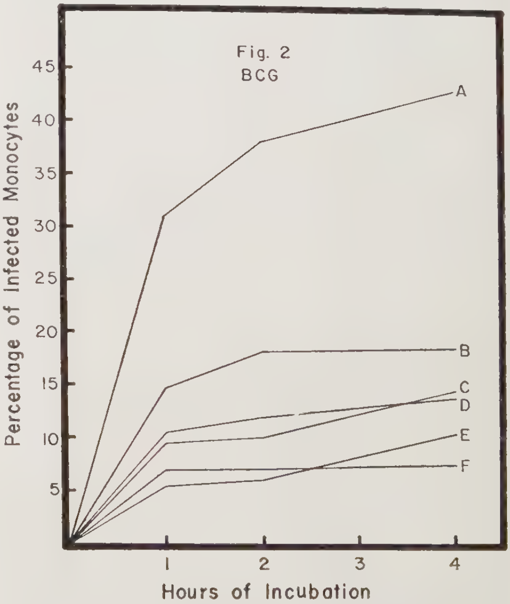

FIG. 2. Phagocytosis of BCG by altered and unaltered mononuclear phagocytes. A—Monocytes with no altering agent; B—monocytes with trypan blue; C—monocytes with cement; D—monocytes with silica; E—monocytes with coal dust; F—monocytes with India ink.

Several reports(6-10,12-14) suggest various reasons for the disseminated nature of tuberculosis in silicotic individuals. Although somewhat limited, these results may suggest that the phenomenon of "blockade" of phagocytic cells could be of importance in helping explain the spread of tuberculosis in individuals exposed to extensive dust inhalation.

Summary. Mononuclear phagocytes which have been altered by ingestion of various particles, take up tubercle bacilli less readily than the same type cell in an unaltered state. This decreased phagocytic activity is observed with both attenuated and virulent tubercle bacilli.

We are grateful to Dr. C. W. Johnson and Dr. W. M. Bright, of the Departments of Microbiology and Anatomy respectively, for their valuable suggestions.

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Tissue Culture Studies on Arbor Viruses of the Japanese B-West Nile Complex.* (23435)

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In October, 1955, a tissue culture section was established at the Virus Research Centre (VRC), Poona, India, with a view to developing tissue culture methods for the study of arthropod-borne (arbor) viruses in India. This communication presents some of the initial results obtained with viruses of the Japanese B-West Nile complex of the B Group of arbor viruses in monkey kidney epithelial cell and chick embryo cell tissue culture. Similar attempts with the Egypt 101 strain of West Nile virus in HeLa, KB, HELL, Detroit 6 (1,2,3,4) and human amnion and chicken kidney cells were unsuccessful in demonstrating significant cytopathogenic effects.

Materials and methods. Viruses: The viruses used in this study were the Egypt 101 strain of West Nile, the P4230 strain of West Nile isolated from the blood of an investigator at the VRC who became ill, presumably from a laboratory infection with West Nile virus(5), the Nakayama strain of Japanese B and the Tamil Nad virus recently isolated from *Culex vishnui* mosquitoes in South India and found to be serologically related to both West Nile and Japanese B(6). Details of the viruses inoculated are given in Table I.

Tissue culture technics: The two types of cell cultures used in this work were *Macacus rhesus* monkey kidney epithelial cells and chick embryo cells. The procedure for preparation of monkey kidney cells was that de-

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* Aid by Dr. Delphine H. Clarke in the preparation of this manuscript is acknowledged with appreciation, as is the technical assistance rendered by Mr. A. R. Diwan, Miss V. S. Chandekar and Mr. A. R. Mendes in carrying out the experiments described.

TABLE I. Data about Viruses Tested in Tissue Culture.

Virus	Source	Passage	Titer, mouse LD_{50}/.03 ml	Types of cells inoculated	Dilution of inoculum
E 101	M 9254	CE_5M_1*	ND	CEC	$10^{-1.3}$
E 101	M 9254	CE_5M_1	$10^{-7.9}$	MKEC	$10^{-1.8}$
P 4230	M 11353	M_6	$10^{-8.5}$	CEC	10^{-1}
P 4230	M 11353	M_6	$10^{-8.5}$	MKEC	10^{-1}
G 2266	M 12001	M_9	ND	CEC	10^{-1}
JBE	M 10838	M_{48}	$10^{-8.6}$	MKEC	10^{-3}
JBE	M 10838	M_{48}	$10^{-8.6}$	CEC	10^{-2}

* CE = chick embryo; M = mouse brain.
ND = not done.

scribed by Rappaport(7) with minor modifications. The chick embryo cell cultures were prepared by a procedure considerably modified from that of Dulbecco(8), the details of which are to appear elsewhere(9). Both preparations consisted of cells trypsinized in 0.2% trypsin in phosphate buffered saline containing penicillin and streptomycin(7) and resuspended after centrifugation in the lactalbumin hydrolysate-Hank's solution-calf serum medium described by Melnick *et al.*(10, 11). The cell suspensions were diluted to a concentration of 0.6-1.0 million cells per ml, as determined by preliminary cell count, and 0.5 ml volumes seeded into tubes. The tubes were incubated in the stationary state at 37°C. In the case of the monkey kidney cells, a good monocell layer was obtained in 6-9 days. Prior to virus inoculation the medium was replaced with lactalbumin hydrolysate-Earle's solution-calf serum(12). The chick embryo cultures attained good growth and were ready for use in 48-72 hours. Prior to virus inoculation the medium was replaced with lactalbumin hydrolysate-Earle's solution-calf serum plus 1 per cent chick embryo extract(9).

Bottle cultures of monkey kidney cells for plaque studies were prepared according to the technic of Hsiung and Melnick(13) with modifications to be described separately(9). The media used were the same as those used for tube cultures. A period of 6-9 days was required to produce a continuous monolayer of cells.

The tube cultures were inoculated with 0.1 ml of various virus dilutions or serum-virus mixtures. The bottle cultures were inoculated with 0.3 ml of virus or serum-virus mixtures. After incubation for 1 hour at 37°C to permit virus invasion, 10 ml of an agar nutrient medium overlay(9) were introduced into each bottle. The optimal conditions for exposure of cells to virus prior to the addition of the agar overlay have yet to be determined.

Following inoculation with virus the tube cultures were incubated at 37°C and examined daily for evidence of cytopathic effect. When such effects were observed the fluid was harvested and inoculated into fresh tubes either undiluted or in a series of 10-fold dilutions. The fluid in the original tubes was replaced with fresh medium. It has been observed that 10^{-2} to 10^{-3} dilutions of harvest fluid produce more rapid and decisive effects than undiluted fluid, a point of some technical importance. Tube cultures of monkey kidney cells were observed daily over a period of 10-15 days. Tube cultures of chick embryo cells were observed over a period of 6-7 days, following which nonspecific degeneration became a complicating factor.

Monkey kidney cells in bottle cultures were incubated at 37°C and observed for plaques from the sixth to the fifteenth postinoculation day. The plaques, visible as color-depleted areas in the neutral red-stained monocell sheet, were counted and recorded every other day.

Tissue culture neutralization tests. These tests were carried out in tube cultures of monkey kidney and chick embryo cells and in bottle cultures of monkey kidney cells, the latter by differential plaque counts. Tenfold dilutions of a titrated frozen or lyophilized tissue culture virus suspension were made extending 2 dilutions beyond the determined tissue culture

ID_{50}. Under exceptional circumstances fresh tissue culture fluid harvests were used. The virus dilutions were made in lactalbumin hydrolysate-Earle's solution-calf serum medium. Specific monkey immune sera and nonimmune control sera from the same animals were mixed without inactivation in quantities of 0.3 ml with 0.3 ml of diluted virus, shaken well and incubated at 37°C for 2 hours, and inoculated in quantities of 0.1 ml in tube cultures and in quantities of 0.3 ml in bottle cultures. Monkey kidney cell culture tubes were examined on the third, fifth, seventh and tenth days after inoculation and chick embryo cell culture tubes daily from the third to the seventh day.

Results. Adaptation of viruses to tissue culture: the general pattern of cytopathogenic effect produced by the Japanese B encephalitis-West Nile group of viruses in tissue culture.

1. *Tubes*: In the early passages in chick embryo cells a few cells showed rounding and ultimate degeneration. This reaction was noticed 3-5 days after inoculation of the virus. In the early passages such cells were scattered among other healthy-looking cells, but in higher passages more and more cells were affected until all were involved. The time between early rounding to complete degeneration was about 48 hours after the onset of the process.

In early passages in tubes of monkey kidney cells, only a few groups of cells appeared granular and distorted 3-5 days after inoculation. These cell groups were more like zigzag lines but in later passages appeared as clumps of 10-20 cells. The distorted cells became granular and rounded 2-4 days after onset of the process and separated from the glass wall. The cells which did not degenerate looked normal in outline but granular. The number of such less affected cells decreased with further passages.

It should be noted that the above-mentioned changes were never observed earlier than 3 days after inoculation, regardless of the virus dose.

2. *Bottles*: Most of the plaque work was done with the P4230 strain of West Nile virus. Early passage plaques were 1-3 mm in diameter with an uneven diffuse outline. The plaques were noticed on the sixth to eighth postinoculation day and there was not much increase in size up to the fifteenth day. The higher passage virus produced round plaques of diffuse outline and larger size—2-5 mm.

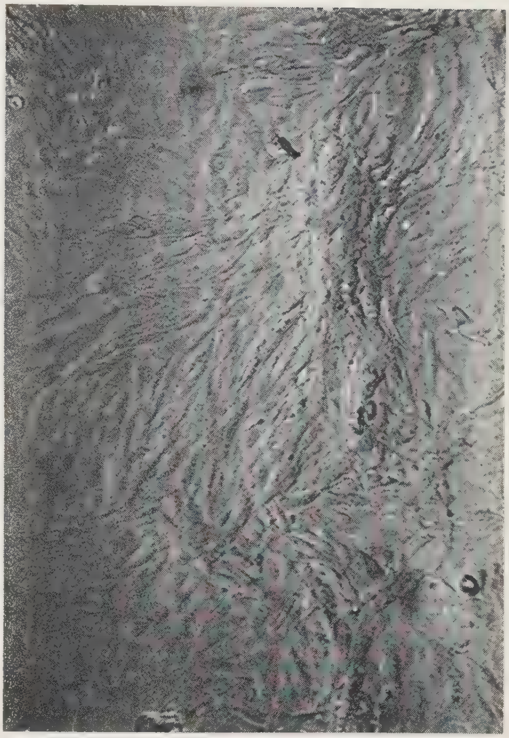
FIG. 1. Normal monkey kidney cells.

P4230 strain of West Nile: This virus gave detectable cytopathogenic effects in tube cultures on the first passage in both chick embryo and monkey kidney cells. Adaptation to chick embryo cells was somewhat more rapid than to monkey kidney cells, so that after 5-6 passages marked pathogenic effect was observed. However, with continued passage the rate and extent of pathogenic effect were enhanced. Cytopathogenicity for monkey kidney cells was well-marked by the 20th passage. Fig. 1 shows normal control monkey kidney epithelial cell growth in a tube for comparison with Fig. 2, which shows the cytopathogenic effect observed in such cultures 3 days after inoculation with 27th tissue culture passage of the P4230 strain. By this passage level plaque formation in bottle cultures was also well-established. The appear-

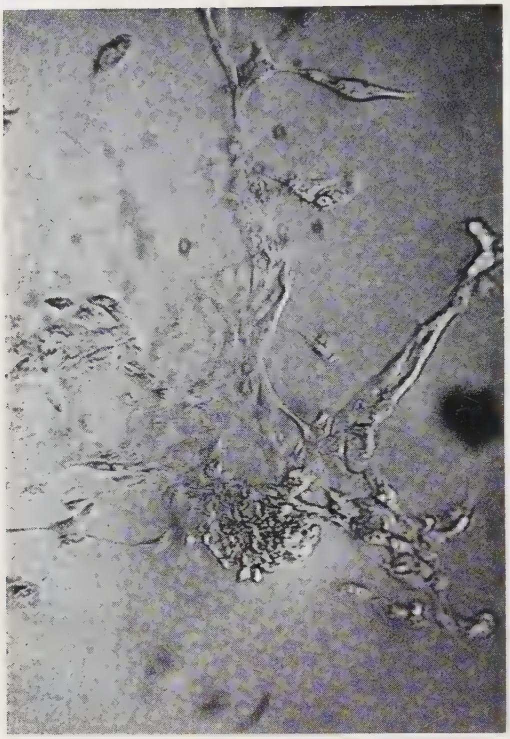

FIG. 2. Monkey kidney cells inoculated with 27th TC passage of P4230 3 days after inoculation with 0.1 ml of $10^{-2.3}$ dilution of the virus.

ance of the plaques 7 days after inoculation with 10^{-3} to 10^{-5} dilutions of 45th passage virus is shown in Fig. 3.

In Table II are presented the results obtained with P4230 virus harvested from 27th passage in monkey kidney cells when titrated in subadult mice (intracerebral inoculation) and tubes of monkey kidney cells. In the same table are shown titrations obtained with P4230 virus at its 23rd passage level in chick embryo tube cultures. These titrations were carried out only in mice and chick embryo tube cultures.

The results of the different methods of assay for infective units can be seen to correlate fairly well. The lower titer achieved by the plaque method can perhaps be attributed to the present lack of knowledge of the optimum conditions for plaque formation.

Egypt 101 strain of West Nile: This strain was readily adapted to chick embryo cells. The 5th chick embryo passage material was tested in monkey kidney cell tube cultures with results similar to those described for the P4230 strain.

Tamil Nad virus: This virus has been studied mainly in chick embryo culture to which it was easily adapted. By the 35th passage it had attained a tube tissue culture titer of $10^{-7.8}$ ($TCID_{50}$/ml) and an adult mouse titer (intracerebral) of $10^{-6.7}$ (LD_{50}/ml).

Japanese B encephalitis virus: Successful adaptation of 3 viruses of the Japanese B-West Nile complex to tissue culture with the development of definitive cytopathogenic effects led to attempts to establish the Japanese B virus in a similar manner. Both chick embryo and monkey kidney cell cultures were used. Early passages of virus into both types of culture resulted in only slight cell destruction in a few scattered areas. However, repeated passage in chick embryo cells led to an increase in degree of cytopathogenicity to the point where tube cultures could be used for virus titrations and neutralization tests.

Cytopathogenicity of this virus for monkey

TABLE II. Comparative Titrations of P4230 Strain of West Nile Virus and Japanese B Virus Adapted to 2-Cell Types in Tissue Culture.

			Titration in	
Virus	Cell type	Tissue culture passage level	Mice, LD_{50}/ml	Tissue culture tubes, $TCID_{50}$/ml
P4230 strain of West Nile	Monkey kidney	27th*	10^{-7}	$10^{-7.5}$
	Chick embryo	23rd	$10^{-6.8}$	$10^{-7.3}$
Japanese B	Monkey kidney	6th	$10^{-8.3}$	$10^{-6.6}$
		25th	$10^{-2.5}$	$10^{-6.3}$
	Chick embryo	5th	—	$10^{-6.5}$
		22nd	$10^{-7.2}$	$10^{-6.0}$

* Endpoint of plaque titration of P4230 virus in bottle cultures of monkey kidney cells was 66 × 10^5.

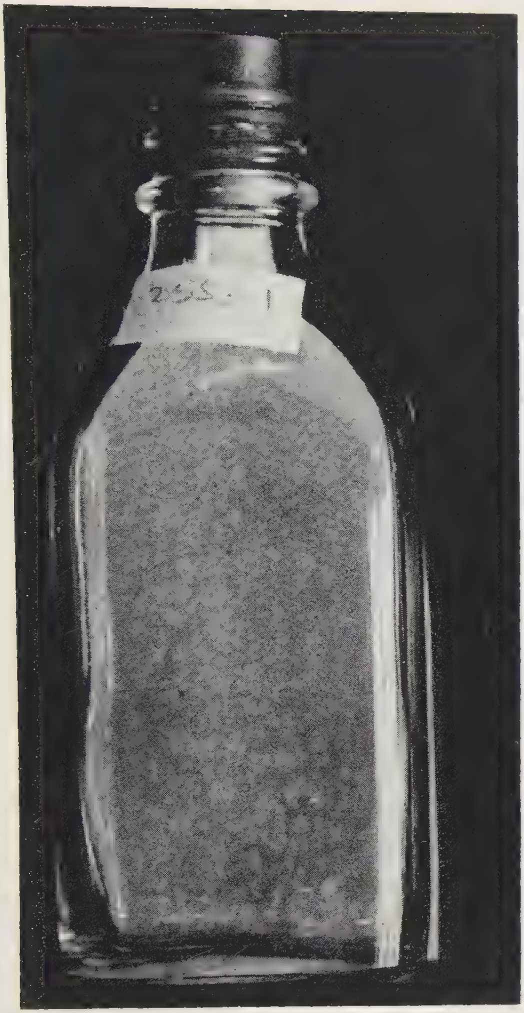

FIG. 3. Plaque formation by P4230 on monolayer of monkey kidney cells 7 days after inoculation with 10^{-3} dilution of virus.

kidney cells was likewise somewhat slow in developing but a marked increase in effect occurred by the 6th passage level and made the performance of tube culture titrations and neutralization tests possible. However, even by the 30th passage satisfactory plaque development had not been achieved, although a few poorly defined plaques were apparent.

An interesting aspect of the process of adaptation of Japanese B virus to monkey kidney cells has been the gradual decrease in the pathogenicity of this virus for mice. This does not seem to be true so far for the virus adapted to chick embryo cells. These results are shown in Table II, which gives comparative assays of infective units obtained by intracerebral titration in subadult mice and by tube culture titration at different tissue culture passage levels. Japanese B immune serum prepared in rhesus monkeys neutralized >2.6 logs of 22nd TC passage virus, demonstrating that it was modification specifically of JBE virus which resulted in the change of mouse pathogenicity after continuous MKEC passage. This neutralization test was carried out in monkey kidney tissue culture tubes.

Neutralization tests: Neutralization tests were done in both types of tube cultures and in monkey cell bottle cultures. The sera used were specific homologous immune sera prepared by inoculation of monkeys with mouse-brain virus suspensions.

Identity of the viruses after passage and adaptation was confirmed by testing in chick embryo and monkey kidney cell cultures, using preimmunization and specific immune sera prepared in rhesus monkeys for the homologous viruses. The monkeys were immunized with mouse-adapted strains which had not previously been cultivated in tissue cultures.

In Table III are shown the results of plaque neutralization tests carried out with the P4230 strain of West Nile virus in monkey kidney cells. The sera used were pre- and postimmunization bleedings from monkeys inoculated with mouse-brain suspensions of the Egypt 101 strain of West Nile and of Japanese B. Also included in the study is the serum obtained from the convalescent laboratory investigator who yielded the P4230 strain of West Nile.

In plaque neutralization Test 2 it will be noted that the preinoculation serum from R 56 produced more than a 10-fold decrease in the plaque count. Presumably this can be related to a nonspecific viricidal effect and indicates one of the complicating factors which must be kept in mind in interpreting the results. Such effects have been observed by various investigators in performing mouse neutralization tests with some of the arbor viruses. More experience will be necessary to determine the frequency of this phenomenon and whether it will prove necessary to eliminate such effects by heating or otherwise

TABLE III. Plaque Neutralization Tests with P4230 Strain of West Nile.

			Plaque counts, PFU/0.3 ml[*]	
Monkey	Immunizing virus	Serum	Test 1[†]	Test 2[†]
R 102	Japanese B	Preinoculation	[‡]	70×10^3
		Postinoculation	$> 45 \times 10^3$ $< 270 \times 10^3$	2.1×10^3
R 24	West Nile (Egypt 101)	Preinoculation	[‡]	180×10^3
		Postinoculation	1.3×10^3	$.5 \times 10^3$
R 56	*Idem*	Preinoculation	[‡]	15×10^3
		Postinoculation	1.05×10^3	0×10^3
	West Nile (P4230)	Human convalescent	$.15 \times 10^3$	0×10^3
		Virus-control	270×10^3	350×10^3

[*] PFU = plaque forming units.

[†] Test 1 carried out with 19th passage and Test 2 with 23rd passage tissue culture virus.

[‡] Confluent plaques at highest dilution tested.

Note: Results of first test are an avg of plaque counts for bottles inoc. with 10^{-2} and 10^{-3} dilutions of virus. Control titer is based on plaque count of 10^{-4} dilution of virus.

Results of second test are an avg of plaque counts in bottles inoc. with 10^{-4} and 10^{-5} dilutions of virus with pre-sera and 10^{-3} and 10^{-4} dilutions of virus with post-sera. Control titer is based on an avg of plaque counts with 10^{-4} and 10^{-5} dilutions of virus.

treating the test sera.

These results served to confirm the identity of the agents producing the cytopathogenic effects and to demonstrate the potential usefulness of the 2 types of technics. The partial neutralization of a strain of West Nile virus by Japanese B immune serum (Table III) is in accord with the known immunological relationship between the 2 viruses first demonstrated by Smithburn *et al.* with the standard intracerebral mouse neutralization test(14).

Summary. The adaptation of 2 strains of West Nile virus, of Tamil Nad virus and of Japanese B encephalitis virus to chick embryo cell and monkey kidney cell cultures is described. After a variable number of passages, cytopathogenicity was sufficiently enhanced that tube cultures could be used for virus titrations with all 3 viruses. Tissue-culture-adapted strains of West Nile virus could be studied by the plaque titration technic in bottle cultures of monkey kidney epithelial cells. This has not been possible so far with Japanese B virus. Neutralization tests with all 3 viruses were successfully performed in tube cultures and the plaque titration method was used for neutralization tests with 1 strain of West Nile. It is believed that the technics used in these studies have potentialities as tools in epidemiological research on West Nile, Tamil Nad, and Japanese B encephalitis viruses and presumably other members of the arbor virus group.

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Factors Influencing Incorporation of Mevalonic Acid into Cholesterol by Rat Liver Homogenates.* (23436)

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A number of studies have established unequivocally that acetate is a precursor of cholesterol. Several other compounds, including pyruvic, acetoacetic, dimethyl acrylic, hydroxy methyl glutaric acids, farnesol, farnesinic acid, and squalene have been suggested as intermediates in the biogenesis of the sterols. Recently the discovery(1), isolation(2), characterization and synthesis(3,4) of a new compound, mevalonic acid (beta-methyl-beta, delta-dihydroxy-valeric acid), which is converted to cholesterol by rat liver homogenates (5) in relatively high yield was announced. Several isotope labeling experiments have now established a mechanism by which the "head to tail" condensation of mevalonic acid units to yield dihydroisoprene chains may be visualized(6,7,8,9). During the course of studies on incorporation of mevalonic acid into cholesterol by rat liver homogenates, a number of conditions and compounds have been observed which influence the extent of the conversion. These findings may be useful to those concerned with sterol biosynthesis.

Methods and materials. The biosynthetic experiments involving conversion of mevalonic acid (MVA) to cholesterol were carried out with liver homogenates by the procedures of Bucher(10,11) as modified by Rabinowitz and Gurin(12) and by Tavormina *et al.*(5,6). Unless specified otherwise, livers from young rats (either sex, less than 150 g in weight, preferably 70-80 g) were rapidly removed following death by decapitation. The livers were cooled in a beaker of ice water. After 5 min the livers were suspended in a small amount of homogenizing buffer and cut into small pieces with scissors. Buffer was then added to a total volume (liver and buffer) equal to 3 times the volume of liver. The mixture was then ground with a loose-fitting Potter-Elvehjem homogenizer (2-3 excursions of the plunger). As is the case with acetate incorporation, incorporation of MVA into cholesterol is also markedly influenced by the clearance between the plunger and bowl of the homogenizer and, as pointed out by Bucher, with acetate, for high incorporation a homogenizer especially ground with a clearance of 0.5 mm should be used. Combined homogenates were centrifuged in a clinical centrifuge at 1500 rpm for 3 minutes and the supernatant (including an intermediate layer) decanted. Aliquots of this suspension were used as the enzyme source. The incubations were carried out in 125 ml Erlenmeyer flasks containing 5 ml of homogenate, 1 mg of ATP, 1 mg of DPN, 1 mg 2-C^{14}-MVA calculated as the DL dibenzylethylenediamine (DBED) salt from which the dibenzylethylenediamine had been removed by extraction with ether from alkaline solution, 1-2 ml of test compound or water, all in a total volume of 7-8 ml. Two preparations of 2-C^{14}-MVA have been used, one (Prep A) containing 4,000 cpm/mg, the other (Prep B), 15,500 cpm/mg. The flasks were then aerated in a stream of oxygen for 25 seconds, stoppered, and incubated for 4½ hours at 37° in a reciprocating water bath (50 oscillations per minute). Operations prior to incubation were carried out at 5°C. Where microbiological assays for MVA in the enzyme experiments were carried out, 0.1 ml aliquots were removed at the end of the experiment, diluted to 10 ml and heated at 100° for 10 min. to yield inactivated preparations. The term "mevalonic acid, % utilization" used in the tables refers to the per cent of added mevalonic acid "used up" during the enzyme reaction as determined by microbiological assay. The incubation mixtures were saponified, the cholesterol extracted with petroleum ether, precipitated with digitonin, the precipitate washed successively with 85% ethanol, acetone-ether(1:2), and finally with ether. The precipitates were suspended in petroleum

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TABLE I. Effect of Age of Rats and Aeration Conditions on Incorporation of Mevalonic Acid into Cholesterol.

Exp.	Size of rats, g	Aeration	Cholesterol, cpm/mg C	Mevalonic acid, % utilization
1	46	Oxygenated and stoppered	1500	100
	46	Open to air	552	100
	339	Oxygenated and stoppered	912	100
	339	Open to air	469	71
2	65	Oxygenated and stoppered	1947	100
	113	*Idem*	376	100
	210	"	699	100
	400	"	159	68

The experiments carried out with a "loose" homogenizer. The 1 mg of MVA (calculated as the DL DBED salt), used as labeled precursor, contained 15,500 cpm (Prep. B).

ether-acetone-ethanol (1:1:1) and plated. Counts were obtained with a gas-flow counter. The activity of the isolated cholesterol is expressed in terms of counts per minute per mg of cholesterol carbon corrected to infinite thinness with a self absorption curve prepared from cholesterol digitonide. Duplicate flasks were used for each experimental condition; variation between duplicates was usually less than 5%.

Microbiological determinations of MVA were carried out with *Lactobacillus acidophilus* (*bifidus*) ATCC 4963 essentially as described by Skeggs *et al.*(1). MVA as the standard was used in the form of the DL DBED salt. Incubation was at 37° for 18 to 24 hours.

The farnesol, squalene, and hydroxy methyl glutaric acid used were commercial preparations. Three samples of farnesinic acid were studied: #1[†], prepared from geranylacetone by the Reformatsky reaction; #2[‡], and #3[§], prepared from nerolidol. Emulsions of farnesol, farnesinic acid, or squalene were prepared in 1% gelatin by dispersion with a tight-fitting homogenizer. Solutions of farnesinic acid were prepared by suspending the compound in water and adding the minimum amount of dilute sodium hydroxide required to affect solution. Hydroxy methyl glutaric acid was dissolved in water and the solution neutralized with dilute sodium hydroxide.

Results. Effect of age. Bloch *et al.*(13) first observed a correlation of activity with age in the incorporation of acetate into cholesterol by rat liver slices. Subsequent investigators in the field have usually specified the use of "young rats," although the use of "adult rats" has sometimes been described. The present biosynthetic experiments were carried out with rats varying in size from 46 g to 400 g. The liver from very young rats is much more active synthetically than that from older rats (Table I). Similarly, microbiological assays for unmetabolized MVA indicated that this factor is completely utilized by homogenates from younger rats, but utilization is not complete with homogenates from older rats.

The present studies indicate that the "dividing line" between high and low incorporation may be about 100 g. Since the same correlation of age with synthetic activity exists with MVA as with acetate, it is suggested that if there is a limiting reaction influenced by age rather than by a general decline in metabolism, this reaction must be between MVA and cholesterol rather than between acetate and MVA.

Effect of method of killing. It has been suggested(14) that rats killed by dislocation of the cervical vertebrae may yield homoge-

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[†] We are indebted to Drs. W. A. Bolhofer and J. M. Sprague, Merck Sharp and Dohme Laboratories, West Point, Pa., for this compound.

[‡] We are indebted to Dr. Gilbert Stork, Department of Chemistry, Columbia University, New York City, for this compound.

[§] We are indebted to Drs. Frank Dituri and Samuel Gurin, Department of Physiological Chemistry, University of Pennsylvania, Philadelphia, for this compound.

TABLE II. Effect of Method of Killing and Aeration Conditions on the Incorporation of Mevalonic Acid into Cholesterol.

Method of killing	Aeration	Cholesterol, cpm/mg C
Dislocation of vertebrae	Oxygenated and stoppered	1445
Idem	Open to air	474
Decapitation	Oxygenated and stoppered	2035
"	Open to air	746

The experiment was carried out with a "loose" homogenizer. The 1 mg of MVA (calculated as the DL DBED salt), used as labeled precursor, contained 15,500 cpm (prep. B).

nates more active than those killed by decapitation. However, under the present conditions rats killed by decapitation yield homogenates at least equal in activity (Table II).

Effect of cofactors. Rabinowitz and Gurin have demonstrated(12) that with acetate as a labeled precursor, ATP (or AMP), DPN, and coenzyme A are required for synthesis of cholesterol by *fractionated* rat liver homogenates. Amdur *et al.* have shown(7) a requirement for ATP, DPN (or TPN or their reduced forms), but not for coenzyme A for synthesis of squalene from MVA by a yeast enzyme preparation. In the present studies with whole homogenates no requirement for added ATP could be demonstrated, although DPN is stimulatory (Table III).

Effect of substrate level. Various levels of labeled substrate from 0.1 mg to 1.0 mg MVA per flask have been tested (Table IV). With an active enzyme preparation derived from the livers of very young rats by the use of a "loose" homogenizer, the incorporation of MVA into cholesterol is essentially a straight line function of the amount of added substrate. With a less active preparation from older rats prepared with a "tight" homogenizer, incorporation is approximately the same at the lowest level, but the extent of incorporation rapidly falls off with increasing substrate level. Despite the fact that with an active enzyme preparation MVA utilization is invariably complete as determined by microbiological assay of the digests at the conclusion of an experiment, it is estimated that under the most favorable conditions studied probably no more than 25-35% of added DL MVA appears in cholesterol.

Effect of oxygenation. Oxygen is required for biosynthesis of cholesterol with the usual enzyme preparations. Under anaerobic conditions the synthesis stops at squalene(15). It was found (Tables I and II) that cholesterol synthesis is much more extensive in flasks where the homogenate was swirled in a stream of oxygen and then stoppered than in

TABLE III. Effect of Cofactors on Incorporation of Mevalonic Acid into Cholesterol.

Exp.	Homogenizer	Substrate activity, cpm	System	Cholesterol, cpm/mg C
1	Tight	4,000 (Prep. A)	Complete	300
			Less ATP	312
			" DPN	236
			" ATP and DPN	232
2	Loose	15,500 (Prep. B)	Complete	1831
			Less ATP	1756

TABLE IV. Effect of Substrate Level on Incorporation of Mevalonic Acid into Cholesterol.

			Substrate		
Exp.	Homogenizer	Size of rats, g	Level, mg	Activity, cpm	Cholesterol, cpm/mg C
1	Tight	150	.1 Prep. A	400	64
			.2	800	95
			.5	2,000	134
			1.0	4,000	144
2	Loose	70	.1 Prep. B	1,550	308
			.2	3,100	603
			.5	7,750	1365
			1.0	15,500	2395

TABLE V. Effect of Various Analogs on Incorporation of Mevalonic Acid into Cholesterol.

Exp.	Homogenizer	Substrate activity, cpm	Analog	Cholesterol		Mevalonic acid, % utilization
				cpm/mg C	% reduction	
1	Tight	4,000 (Prep. A)	None	171		94
			Farnesol, 4 mg (emulsion)	20	88	85
			Farnesinic acid #1, 4 mg (")	0	100	64
			Squalene, 4 mg (")	133	22	90
2	"	*Idem*	None	485		98
			Farnesol, 4 mg (")	68	86	98
			Farnesinic acid #1, 4 mg (")	0	100	94
			Squalene, 4 mg (")	384	21	98
3	"	"	None	80		58
			Farnesinic acid #1, 1 mg (solution)	3	96	36
			Hydroxy methyl glutaric acid, 1 mg (")	80	0	58
4	"	15,000 (Prep. B)	None	209		
			Farnesinic acid #1, 2 mg (")	10	95	
			" " #2, 2 " (")	13	94	
			" " #3, 2 " (")	11	95	
5	Loose	*Idem*	None	2000		
			Farnesinic acid #1, 1 mg (")	1352	32	
			" " #2, 1 " (")	1349	32	
			" " #3, 1 " (")	1022	48	

flasks that were not oxygenated but were left open to the air. Whether or not the results obtained are a function of oxygen tension alone has not been determined.

Effect of various postulated cholesterol intermediates. Bloch(16) observed that when squalene, 7-dehydrocholesterol, Δ 7-cholestenol, or cholesterol itself is incubated with rat liver slices along with C^{14}-acetate, the counts found in isolated cholesterol are markedly reduced. On the other hand, incubation with a number of other sterols, farnesol, farnesal, farnesinic acid or nerolidol did not influence the cholesterol counts. He interpreted these findings to mean that 7-dehydrocholesterol, Δ 7-cholestenol, and squalene are preferentially incorporated into cholesterol over acetate and are therefore intermediates in cholesterol biogenesis. The negative results obtained with the second group of compounds were interpreted as indicating that these compounds are not intermediates. Using essentially the same technic except that fractionated rat liver homogenates rather than liver slices were employed and the labeled precursor was a biologically-active impurity contained in a preparation of beta-hydroxy-beta-methyl glutaric acid, Dituri *et al.*(17) found that the incubation of labeled precursor with farnesol or farnesinic acid led to the isolation of squalene markedly less active than that found in the absence of either of these compounds. Added carrier farnesinic acid was re-isolated from the incubation mixtures and was found to contain a small but detectable amount of label. No label was found, however, with carrier farnesol. These investigators concluded that farnesol and farnesinic acid are intermediates in the biosynthesis of squalene. Evidence that farnesinic acid is not a precursor of cholesterol has been presented by Sandermann and Stockmann(18), who synthesized C^{14}-labeled farnesinic acid by the Reformatsky reaction from geranylacetone and found that the compound is not a precursor of cholesterol in rat liver. Insufficient data are given, however, for a critical evaluation of the work.

In the present studies, when farnesol, farnesinic acid (all 3 samples), or squalene is incubated with rat liver homogenates along with 2-C^{14}-MVA, the counts found in cholesterol isolated from the digests are markedly reduced over the values found in the cholesterol from control digests. (Table V). Similarly, MVA utilization, as determined by microbiological assay of the factor remaining after the period of synthesis, was reduced by the analogs. Of the 3 compounds studied, farnesinic acid is the most active. The effect was observed both when farnesinic acid was present in an emulsion and when it was present in true solution as the sodium salt. The effect observed with farnesinic acid was demonstrable both with a "highly active" enzyme preparation and with "less active" preparations. Farnesol and squalene were less active than farnesinic acid. Neither of the latter 2 compounds could be studied in aqueous solution. Hydroxy methyl glutaric acid, at least in comparable amounts, was found to be inactive with respect to both cholesterol biosynthesis and MVA utilization.

It is appreciated that a variety of interpretations may be applied to the results obtained with these analogs since a similar effect in influencing the incorporation of mevalonic acid would be expected with a compound that is either a precursor or an antimetabolite. With respect to squalene, independent evidence that squalene is a precursor of cholesterol seems so overwhelming that the effect observed here probably means that squalene competes with MVA for incorporation into cholesterol. The results obtained with squalene, therefore, are probably attributable to a precursor effect and the role of squalene in biogenesis of cholesterol is not questioned. As far as farnesinic acid is concerned, the influence of the compound on acetate incorporation is controversial, but the counts found in squalene from the impurity contained in hydroxy methyl glutaric acid or in cholesterol from MVA in the present work are markedly reduced by the compound. In the absence of labeled analog it is impossible with the liver technic employed to determine whether a metabolite or antimetabolite effect is operative. Separate studies have shown that in a system involving utilization of MVA by *Lactobacillus acidophilus* farnesinic acid is a definite antimetabolite(19). The fate of MVA in the lactobacillus is at present unknown and it is conceivable that farnesinic acid is a metabolite in one system and an antimetabolite in another. Until more conclusive results are obtained, it seems reasonable to question the role of farnesinic acid as an obligatory intermediate in cholesterol biosynthesis. The data obtained with farnesol are more difficult to interpret. While the work described herein was nearing completion, it was reported(20) that the feeding of farnesol to rats failed to influence the cholesterol content of the liver, nor did the prior administration of farnesol influence cholesterol synthesis from administered 1-C^{14}-acetate. On the other hand, the feeding of squalene led to the accumulation of excess cholesterol in the liver and less synthesis of cholesterol from administered acetate. Possibly farnesol is a weak antimetabolite of cholesterol biosynthesis.

Summary. Incorporation of mevalonic acid into cholesterol by rat liver homogenates is favored by (a) use of tissues from very young rats, (b) use of a "loose" homogenizer, and (c) saturation of the homogenate with straight oxygen and stoppering. Incorporation of mevalonic acid is less in the presence of compounds that may be intermediates or antimetabolites of cholesterol biosynthesis. With whole homogenates biosynthesis is not markedly influenced by the method of killing the rats or by additions of ATP.

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Cataracts Following Mumps Virus in Early Chick Embryos.* (23437)

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Clinical reports dealing with the effect on the human fetus of German measles (rubella) in the mother, stressed the occurrence of cataracts along with heart damage, deafness, mental retardation and other defects. Other viruses have been suspected of being etiologically related to congenital defects and mumps virus has featured prominently among reports of such cases(1,2). Experimental work on the developing chick embryo in this laboratory has shown that infection with Newcastle disease and influenza A viruses resulted in specific damage to the developing lens, the extent depending on method of inoculation, stage of development of the embryo and virus titer of the inoculum(3-7). While these effects were definite and have been amply confirmed, these viral infections in the embryo were lethal within 48 hours. More recently, inoculation of the developing chick embryo with the Enders strain of mumps virus produced effects which were compatible with survival of many of the embryos for 3 to 15 days after inoculation and prominent in the results was the presence of cataracts.

Materials and methods. Virus preparations. The Enders strain of mumps virus, obtained from the American Type Culture Collection, was inoculated in 10^{-3} dilution into the allantoic cavities of 8-day chick embryos. The infectious allantoic fluids, harvested after incubation at 35°C for 7 days, served as stock virus suspension. In order to assure a high titered preparation, the fluid from each egg was harvested separately and tested for hemagglutinating activity at 1-100, 1-500 and 1-1000 dilutions. Much variation was observed, ranging from no hemagglutination to a titer of 1-1000. Fluids showing titers of 1-500 or more were pooled and dispensed in ampoules for storage at –70°C. For inoculating into early chick embryos the suspensions were diluted in buffered saline (pH 7.2) to the required infectivity. In some preparations 500 units of penicillin and 100 μg of streptomycin per ml were added to control bacterial growth. However, earlier mortality was noted when antibiotics were added to the mumps virus inoculum. In most instances, therefore, no antibiotic was added so as to eliminate the possibility that changes in the embryo were the result of a possible synergistic action of antibiotics and virus.

Infectivity titrations. Infectivity titers were determined by inoculating 10-fold dilutions of the virus suspensions into the allantoic cavities of respective groups of 8-day-old embryos. These were tested after 7 days for virus growth as indicated by hemagglutinability of the allantoic fluids. The ID_{50} was calculated by the method of Karber(8). Young embryos to be tested for rise in virus titer were harvested whole, and ground in a Ten Broeck tissue grinder. A portion of tissue was then measured by volume and sufficient

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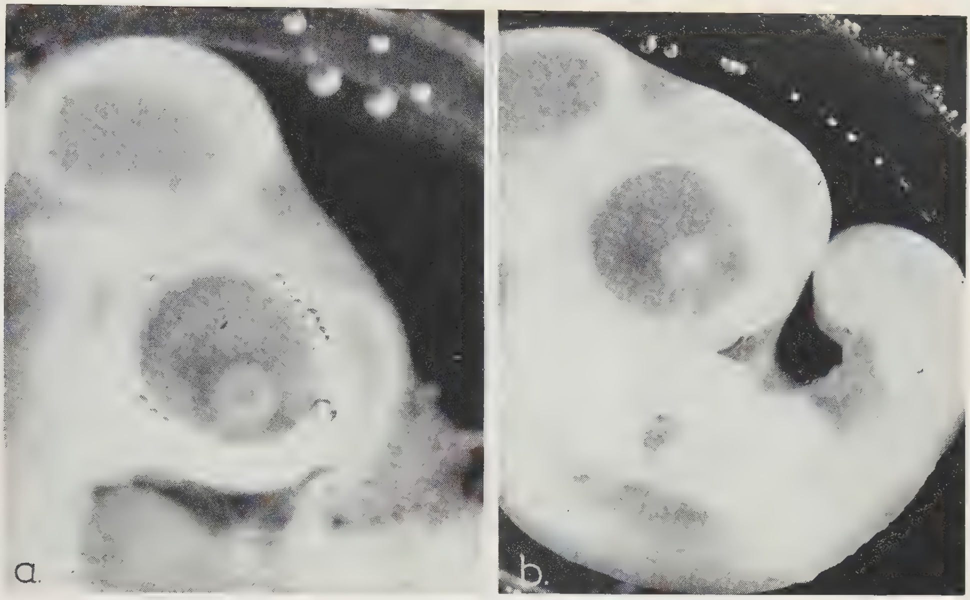

FIG. 1. a. Optic cup and lens of a control chick embryo of 5 days incubation. b. Lens opacity in a 5 day chick embryo inoculated 3 days previously with mumps virus.

saline added to make the initial 1-10 dilution.

Inoculation of embryos. Eggs incubated in a forced draft incubator at 98-99°F for approximately 48 hours were in stages of development from 10-14 according to Hamburger and Hamilton(9). Such embryos were prepared with openings and inoculated under the vitelline membrane and over the blastoderm (4) with 0.05 ml amounts of virus or control suspension. *Controls.* Embryos of the same incubation settings were inoculated with normal allantoic fluids in dilutions equal to those of the experimental groups. *Examination of embryos for abnormalities.* Embryos were examined daily under 24 power magnification by observation through the shell openings. Since development of the membranes obscured the view of the older embryos, in some experiments the embryos were harvested at appropriate intervals after inoculation for direct examination in the unfixed state.

Experimental procedure and results. Preliminary studies showed that embryos of 48 hours incubation which received $10^{5.7}$ to $10^{6.9}$ ID_{50} of mumps virus, lived from 1-15 days after inoculation. Observation during the first 2 days revealed a few slight axis twists and some retardation of the amnion in the mumps infected group. By the third or fourth day, definite retardation of the amnion in the infected group was observed and permitted a view of many embryos which otherwise would be obscured by normal development of the membranes at this age. The developing crystalline lens of a significant number of these embryos showed areas of dense white opacity, centrally or excentrally located (Fig. 1). On daily observation, no regression was ever observed, the cataracts tending rather to increase in size. Experiments in which surviving embryos were harvested on the fifth day postinoculation, showed that the cataracts occurred either bilaterally or unilaterally, the unilateral ones involving chiefly the right lens. Other developmental deviations appearing about the same time included failure of the lower body axis to turn onto the left side resulting in mild abnormalities of axial torsion and in a "spraddling" of the lower limb buds on the yolk sac. Of 104 embryos receiving mumps virus, 60 lived 3 days or longer and in 46 embryos cataracts were observed grossly *in vivo.* Of 80 control embryos, 71 survived 3 days or longer and were harvested at intervals up to the fifteenth day after inoculation which was the longest time that a mumps-inoculated embryo survived. None showed cataract. In some of the longer surviving mumps-

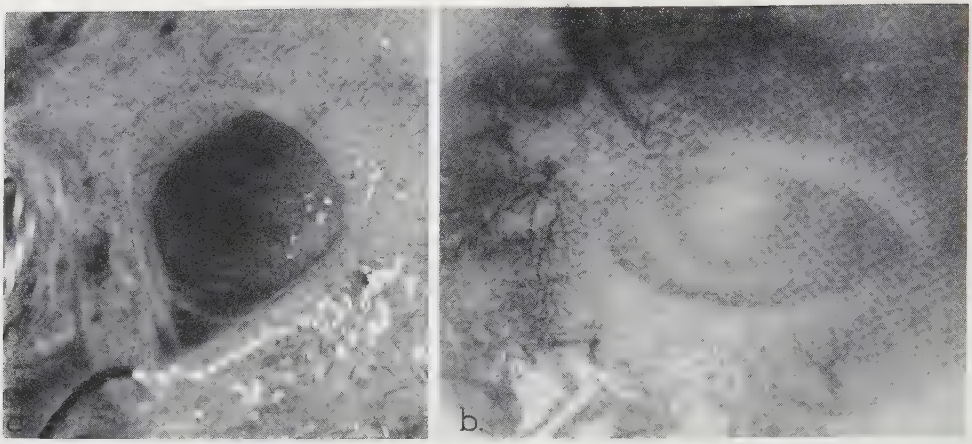

FIG. 2. a. Lens of a control chick embryo of 17 days incubation. b. Cataract in a 17 day chick embryo inoculated 15 days previously with mumps virus.

inoculated embryos there was evidence of other damage, such as irregularity of pigmentation of the choroid layer of the optic cup, hemorrhage into the lens and retarded development of the lids and skin around the eye, as well as a general retardation of feathering. The 2 longest surviving embryos died at 12 and 15 days after inoculation. Both embryos were very small, being approximately half the size of controls, and both showed cataract (Fig. 2).

Microscopic examination of lenses showing cataract at 3 days and 5 days after inoculation revealed extensive vacuolization and necrosis of the developing lens fibers (Fig. 3).

Effect of virus titer of inoculum. Embryos receiving $10^{3.8}$ ID_{50} or less virus did not show the severe amniotic retardation or the axis abnormalities which were characteristic of embryos receiving the larger amounts of virus. Data presented in Table I show that the incidence of embryos with cataract decreases with dilution of the virus inoculum. Since the embryos were harvested for examination on the fifth day after inoculation the results of these studies are comparative.

TABLE I. Production of Cataracts by Mumps Virus in Chick Embryos of 48 Hours Incubation.

Inoculum	Eggs inoc.	Embryos surviving 5 days post-inoc.	Surviving embryos showing cataract
			%
Mumps $10^{5.8}$ ID_{50}	78	33	28 (85)
" $10^{3.8}$ ID_{50}	45	31	10 (32)
" $10^{1.8}$ ID_{50}	45	37	2 (5)
Heated mumps*	43	33	0

* Mumps suspension of titer $10^{5.9}$ was heated one hr at 60°C.

Increase of virus titer in affected embryos. Groups of embryos of 48 hours incubation, inoculated with $10^{5.8}$ ID_{50} of virus, were harvested at 3 hours after inoculation and thereafter at daily intervals. Hemagglutination tests of the ground tissues showed an increase in titer from less than 1-40 at the 3 hour interval to a titer of 1-320 at the 2-day interval. Infectivity titrations on the ground tissues also showed an increase in virus titer up to the second day after inoculation, at which time a titer of $10^{6.7}$ ID_{50} per 0.5 ml was obtained, a 16-fold increase over the titer obtained at the 3-hour interval. Thereafter the infective titer dropped to $10^{5.3}$ where it remained for the next 3 days. The decline in titer was not necessarily due to a decrease in virus production. Since the tissues of the embryo are growing rapidly during this period, the apparent decline in virus titer may have been due to a change in ratio between tissues not directly accessible to the virus, *i.e.*, mesodermal and entodermal tissues, and those directly exposed to the virus under inoculation procedures employed, *i.e.* ectoderm and organs differentiating from it at the time of inoculation. A change in susceptibility of various tissues as differentiation proceeds could also be a factor. It is clear, however, that multi-

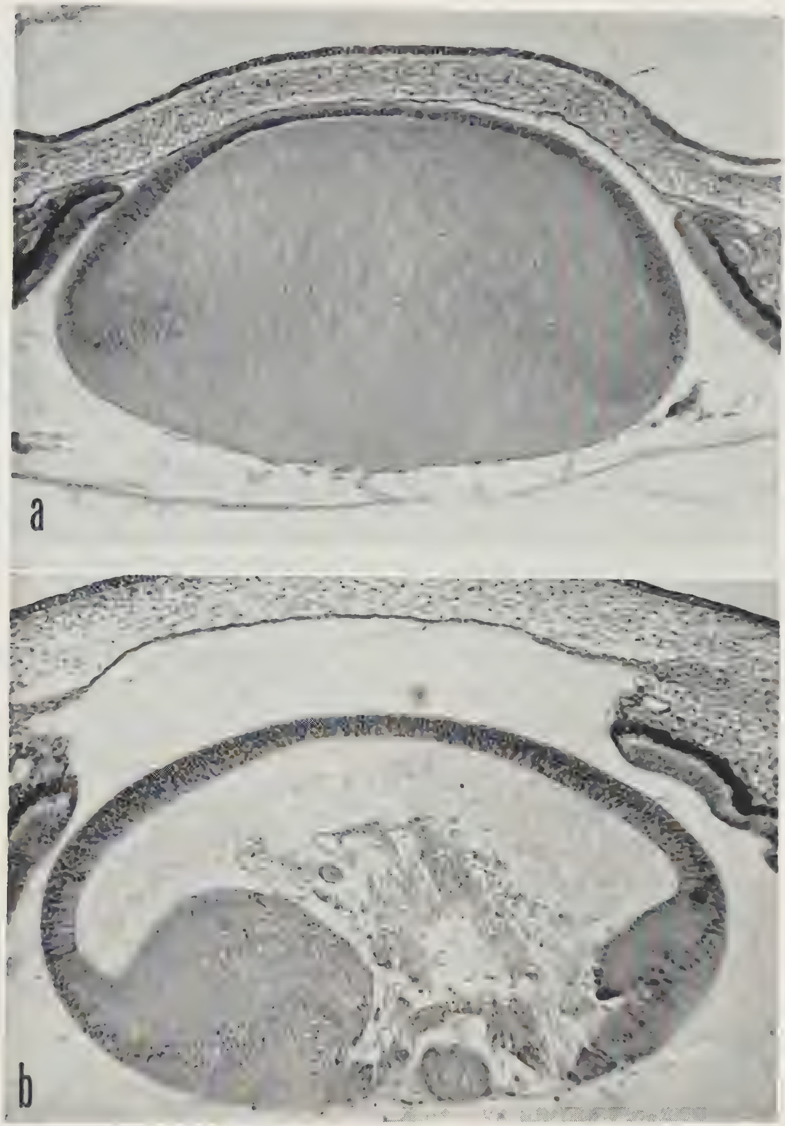

FIG. 3. a. Microscopic section of normal lens of a 7 day chick embryo. b. Microscopic section of a lens showing cataract in a 7 day embryo inoculated 5 days previously with mumps virus.

plication of the mumps virus occurs in chick embryos of this age even after inoculation of high titered virus inoculum. This is of interest since such a high titer of virus appears to be needed for optimal yield of defective embryos.

Effect of heat inactivated virus. An allantoic fluid suspension of mumps virus diluted in buffered saline to contain $10^{5.8}$ ID_{50} per 0.05 ml, was divided into 2 portions, one remaining untreated while the other was heated 1 hour at 60°C. The preparations were then inoculated into respective groups of 48-hour embryos. Table I shows that heating destroyed the capacity of the virus to produce cataracts. No virus was recovered from the heated preparation when it was inoculated into the allantoic cavity of 8-day embryos.

Discussion. A variety of defects has been reported following virus infections of the early chick embryo, ranging from axis twists and collapsed encephalon following influenza A

(10,11), herpes and vaccinia(12) to specific defects such as absence or retardation of such organs as the lens, auditory vesicles or caudal neural tube following Newcastle disease and influenza A viruses(3-7). Earlier reports of the effects of mumps virus in early chick embryos(10) described no characteristic defects but reported a slight increase of various nonspecific changes of types commonly observed in the controls. The finding of cataracts and other defects in the present study may be accounted for by a difference in properties among strains of mumps virus, or may be due to variations in experimental technics employed.

It is of interest to observe that, in this laboratory, three viruses, all members of the myxovirus group, produced quite different effects when inoculated in high titer in young chick embryos under similar experimental conditions. Influenza A, within 24 hours, produced axis twists and collapsed encephalon as well as specific changes in development of the lens, auditory vesicles or neural tube. Newcastle disease, within 24 hours, produced only specific changes in the lens, auditory vesicles and neural tube. Mumps, however, inoculated in equally high titer produced no grossly observable defects within 24 hours. Only after 72 hours were mild axis twists and lens opacities observed. Although these different effects may reflect fundamental differences in cell-virus relationships, the slower growth of mumps virus, as observed in older embryos, may constitute another factor. A review of the results of virus-induced defects in chick embryos indicates that viruses producing the most severe defects also produce rapid death when inoculated into early embryos. On the other hand, the mumps virus, producing relatively mild defects, permits much longer survival of the embryos. This is in agreement with the impression that, in the human, the more virulent maternal infections tend to produce early abortion, whereas milder infections may permit survival of a congenitally defective infant.

The similarity of ocular changes produced by the mumps virus in the chick embryo and those observed with rubella infection in the human subject, suggests that further studies at the microscopic level be carried out in the chick embryo to ascertain whether the heart, brain and auditory systems are also damaged, as has been reported in the rubella syndrome.

Summary. Mumps virus, inoculated into embryonated chicken eggs of 48 hours incubation, produced a high percentage of cataracts in embryos surviving from 3 to 15 days after inoculation. Other defects observed included general growth inhibition, retardation in development of the amnion, mild axis twists, retardation of feathering, defects of the choroid layer of the optic cup and retarded development of the lids and skin around the eye. A decrease in the percentage of embryos showing defects was directly related to a decrease in the titer of the virus inoculated. Virus inoculated into the young embryos was shown to increase in titer over a period of 2 days after inoculation and was recoverable in high titer over a period of at least 5 days. Heat-inactivated mumps virus produced no cataracts or other defects.

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Vit. B_{12} in Thyrotoxicosis and Myxedema.[*] (23438)

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Thyroactive materials fed to animals enhance their vit. B_{12} requirement. This procedure is used in vit. B_{12} assays on chicks to increase the sensitivity of the test(1). Information on how hyper- and hypometabolic states affect B_{12} requirements in man is lacking. We have studied the acute blood responses and urinary excretion of vit. B_{12} in normal, hyperthyroid and myxedematous subjects after an intramuscular (IM) load dose of 50 μg B_{12}.

Materials and methods. The subjects were 6 normal, 10 hyperthyroid and 4 myxedematous patients seen in the endocrine and thyroid clinics and the medical wards of Mount Sinai Hospital. The diagnosis of hyperthyroidism was made clinically and corroborated by the 24-hour radioactive iodine (I^{131}) uptake by the thyroid gland and the protein-bound I^{131} levels in the blood(2) (Table I). Two of the myxedematous subjects (M.S., S.C.2) had undergone surgery for hyperthyroidism, one (F.L.) developed hypothyroidism as a result of treatment with radioactive iodine, and one (R.H.) had myxedema secondary to panhypopituitarism. The normal subjects had no evidence of thyroid disease or chronic debilitating illnesses. No subject was taking vitamin preparations. Diet was not controlled, except that on the day of vit. B_{12} administration the subjects were asked to omit breakfast and to eat a small luncheon. For control purposes, 10 ml of blood were drawn from the antecubital vein of each subject. Fifty μg vit. B_{12} were then given IM and blood was withdrawn at 4- and 8-hour intervals. Urine was collected for 8 hours following vit. B_{12} administration. Whole blood and urine were assayed for vit. B_{12} with *Euglena gracilis*(3,4).

Results. As summarized in Table II, the blood levels of B_{12} at zero ("control") time for the normal and myxedematous subjects were almost the same; 575 and 600 μμg/ml respectively. The hyperthyroid group had a lower control vit. B_{12} blood level with a mean of 320 μμg/ml. There were marked differences in the blood levels of the 3 groups 4 hours after B_{12} administration. The smallest rise was observed in the hyperthyroid group (mean 615 μμg/ml), which was approximately ½ the rise observed in the normal subjects and ⅓ the rise noted in the myxedematous group. At the end of 8 hours, these ratios were still maintained, although the hyperthyroid group had returned to almost the control levels or lower values (Table II).

The 8-hour urinary B_{12} excretion of the 3 groups showed a mean for the normal subjects of 8.2 μg and ½ this for the thyrotoxic group; the hypothyroid group excreted 2 times as much as the normal group.

Discussion. The values for vit. B_{12} given in this study are for whole blood and are 40-

TABLE I. Laboratory Data on Thyrotoxic Subjects.

I^{131} uptake, % tracer dose retained in 24 hr	PBI^{131}, % tracer dose/l plasma, 72 hr[‡]	Estimated duration of toxicity
80	1.50	8 yr[*]
70	1.24	2 "
90	.61	2 "
90	1.01	1 "
78	2.10	1 "
57	1.10	1 " [†]
90	.72	5 "
80	1.00	6 mo
70	.29	2 "
84	.36	2½ yr[†]

[*] Subject treated with propylthiouracil from 1950-1952, and relapsed in 1956.

[†] Nodular goiters; all other subjects had diffuse goiters.

[‡] Normal values of PBI^{131} are 0.26% or less of tracer dose/l of plasma.

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[*] Aided by grants from U. S. Public Health Service and National Assn. for Mental Health to Scottish Rite Research Com.

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[‡] Fellow of Dazian Fn. for Medical Research.

TABLE II. Blood and 8 Hour Urinary Excretion of Vit. B_{12} Levels following a 50 μg IM Load Dose.

Name	0 time, μμg/ml	4 hr, μμg/ml	8 hr, μμg/ml	8 hr urine, μg
Group 1. Normal subjects				
L.M.	450	825	785	8.6
H.Z.	435	705	705	†
A.G.	515	840	600	†
C.Z.	500	1265	690	12.7
E.F.	615	1550	800	9.1
L.M.R.	850	1445	1150	3.8
S.C. 1*	650	1850	1050	6.8
Mean	575	1210	825	8.2
Group 2. Myxedematous subjects				
S.C. 2*	450	1350	1150	20.7
R.H.	450	1700	1400	†
F.L.	760	1250	710	27.5
M.S.	730	2400	2000	7.7
Mean	600	1675	1315	18.6
Group 3. Thyrotoxic subjects				
B.G.	150	200	170	2.9
S.H.	115	200	115	1.7
G.R.	185	330	260	8.4
B.B.	200	550	400	2.1
N.G.	210	475	305	.9
J.B.	220	735	685	4.9
R.C.	350	600	310	3.5
F.N.	450	505	575	3.9
P.S.	525	1000	750	2.9
M.S.	810	1475	520	8.9
Mean	320	605	410	4.0

* S.C. 1, prior to therapy; S.C. 2, three wk after therapy with 100 μg L-triiodothyronine/day.

† Specimen accidentally discarded or not completely collected.

60 μμg/ml higher than those reported for serum(5). It would appear that some of the control blood levels of the hyperthyroid subjects (Table II) approach those seen in untreated pernicious anemia(6).

Blood levels and urinary excretion of vit. B_{12} accurately reflect the intensity of utilization of vit. B_{12}(7,8). As seen in the present study, hyperthyroidism is associated with (*a*) lower initial B_{12} blood levels, (*b*) a small rise after B_{12} administration, and (*c*) low urinary excretion of the vitamin. These results suggest a greater retention and utilization as compared with normals. The myxedematous group had the highest B_{12} levels and greatest urinary excretion *i.e.* the smallest retention and utilization of the vitamin.

The flagellate *Ochromonas malhamensis* has a B_{12} requirement similar to that of higher animals(9,10). When it is subjected to heat stress, its B_{12} requirement is greatly increased (11). This suggests the analogy that the B_{12} system in higher animals is easily upset or inefficient under conditions of metabolic stress.

Summary. The whole-blood B_{12} values in 10 hyperthyroid subjects before and after a load dose of B_{12} were lower than for a normal and a myxedematous group. The mean 8-hour urinary excretion after B_{12} administration in hyperthyroid subjects was considerably lower than in the normal and myxedematous groups. These data suggest that B_{12} turnover and demand is appreciably greater in hyperthyroidism and appreciably lower in myxedema.

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Isocitric and 6-Phosphogluconic Dehydrogenases in Human Blood Serum.

(23439)

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Although a number of dehydrogenases utilizing DPN† as a hydrogen acceptor have been demonstrated in the blood serum(1-4) and cerebrospinal fluid(5) of mammals, no information concerning TPN-linked enzymes in mammalian extracellular fluids is extant. Faulkner(6) found recently that silkworm blood contains a "malic" enzyme(7) and a polyhydric alcohol phosphate dehydrogenase both of which are specific for TPN.

We have found that both the serum and the erythrocytes of human and rat blood contain isocitric dehydrogenase (ICD):

Isocitrate + TPN $\rightleftharpoons$ α-Ketoglutarate + CO_2 + TPNH + H^+

and 6-phosphogluconic dehydrogenase (PGD):

6-Phosphogluconate + TPN $\rightleftharpoons$ Ribulose-5-phosphate + CO_2 + TPNH + H^+.

These enzymes are similar insofar as they both catalyze the reversible oxidative decarboxylation of their substrates and require TPN as a specific hydrogen acceptor. The properties of these enzymes in human blood serum were found to be similar to those reported for other animal tissues(8,9). Recent reports of large changes in activity of a number of glycolytic enzymes and transaminases of serum in certain diseases(2,3,10,11) prompted us to develop methods for determination of ICD and PGD in serum, which are here reported.

Methods. Human blood samples were drawn from the cubital vein without regard to the fasting state. The blood was transferred to polyethylene centrifuge tubes and allowed to clot at room temperature. The serum was separated by centrifugation, removed with a dropping pipette, and centrifuged again to remove all traces of erythrocytes. The millimolar extinction coefficient of reduced pyridine nucleotides at 340 mμ was assumed to be 6.22. PGD of rat liver was purified according to Glock and McLean(12); the preparations were devoid of glucose-6-phosphate dehydrogenase activity. ICD was purified from rat heart by the method of Ochoa(13). All spectrophotometric measurements were made with a Beckman model DU spectrophotometer with either quartz or corex cells of 1 cm light path. In most experiments the temperature of the cell compartment was maintained at 25° by circulating water at this temperature through thermospacers. Total serum protein was estimated by the quantitative biuret method(14). *Determination of ICD activity.* For each determination a blank and an experimental cell was used. Both cells contained 0.5 ml Tris buffer pH 7.5 (0.1 M); 0.2 ml TPN (0.001 M) dissolved in 0.15 M NaCl and 0.3 ml $MnCl_2$ (0.01 M) dissolved in 0.15 M NaCl. The blank cell contained 0.05 ml 0.15 M NaCl and the experimental cell contained 0.05 ml of sodium DL-isocitrate (0.1 M). Usually these components were mixed in small tubes placed in a constant temperature bath at 25°. The reaction was initiated by the addition of 0.5 ml of serum to each tube. The components were mixed by inversion 3 times, the ends of the tubes being covered with a sheet of "parafilm". Immediately after mixing, the reaction mixtures were poured into the spectrophotometer cells. The absorbency of the experimental cell at 340 mμ versus that of the blank cell was measured at 3- to 5-minute intervals for 12 to 20 minutes. Sera with abnormally high ICD activities were diluted with 0.15 M NaCl just prior to their addition to the reaction mixture. ICD activities were calculated from zero order portions of plots of the change in absorbency of the experimental cell versus time. The activity was expressed in terms of millimicromoles of TPNH formed per 1 ml of serum per hour at 25°. In those experiments where the temperature at which the reaction was run was other than 25°, the activities were corrected to the latter temperature, assuming a temperature coefficient (Q_{10}) of 1.7 over the range of 20°-30°. *Determination of PGD activity*. Both the blank and the experimental cells contained 0.1 ml Tris buffer pH 7.5 (0.5 M); 0.05 ml $MgSO_4$ (1 M); 0.1 ml TPN (0.004 M) dissolved in 0.15 M NaCl and 0.1 ml cysteine HCl (0.1 M, neutralized to pH 7.5 with $NaHCO_3$ just prior to use). The blank cell contained 0.7 ml 0.15 M NaCl while the experimental cell contained 0.6 ml 0.15 M NaCl and 0.1 ml of sodium 6-phosphogluconate (0.01 M). The reaction was initiated by the addition of 0.5 ml of serum to both tubes. The spectrophotometric method for the determination of substrate dependent formation of TPNH, and the method of expression of enzymatic activity was the same as that used for the determination of serum ICD.

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† The following abbreviations are employed: TPN = triphosphopyridine nucleotide; TPNH = dihydrotriphosphopyridine nucleotide; DPN = diphosphopyridine nucleotide; DPNH = dihydrophosphopyridine nucleotide; Tris = tris (hydroxymethyl) aminomethane.

Results. Properties of ICD and PGD in blood. Under the experimental conditions described, the rate of substrate dependent formation of TPNH obeyed zero order kinetics in both test systems until more than 50% of the added TPN was reduced. With both enzyme systems the initial rate of reaction was strictly proportional to the quantity of serum added. The rate of reduction of TPN in the absence of added substrate by the sera of apparently healthy adults was found to be negligible in comparison with the activity of ICD and PGD. No change in absorbency at 400 mμ was observed in either test system.

The concentrations of TPN, divalent cations and substrates employed appeared to be optimal insofar as increasing them 2-fold did not alter rate of reaction. Cysteine activated the serum PGD of normal humans from 3- to 5-fold at the concentrations used. This thiol was without influence upon serum ICD activity.

The ICD and PGD of normal human serum were found to be specific for TPN. Replacement of this nucleotide by DPN did not lead to a substrate-dependent formation of DPNH. A number of experiments were performed to detect pyridine nucleotide transhydrogenase (15) in serum. In these experiments, the TPN concentration in the ICD test system was reduced by a factor of 10, and the reaction allowed to proceed until all the TPN present (0.02 μmole) was reduced. The absorbency at 340 mμ remained constant after that time. DPN (1 μmole) was then added to both the blank and the experimental cells. No further change in absorbency at 340 mμ occurred, from which it was concluded that pyridine nucleotide transhydrogenase was absent from serum.

The ICD activity of serum was negligible in the absence of added Mn^{++} ions. When added in 10-fold molar excess, Mg^{++} ions permitted approximately 60% of the rate of reaction observed with Mn^{++} ions. With some sera, the formation of TPNH in the ICD test system showed a definite lag period for about the first 3 minutes. This lag period could be diminished by doubling the amount of Mn^{++} added, a procedure which did not affect the rate of reaction once the initial lag period was overcome. It must be emphasized that all reaction rates were calculated from the linear portion of plots of change in absorbency versus time. Mg^{++} ions induced definite but variable activations of serum PGD and hence were added to the test system for this enzyme.

When ICD purified from rat heart muscle was added together with serum to the ICD test system, the increase in rate of formation of TPNH was strictly additive. However, it must be mentioned that erratic results were obtained in attempts to measure the ICD activity of highly lipemic sera, and in these cases, the further addition of purified ICD did not result in the expected increase in activity. With normal sera, the addition of purified rat liver enzyme leads to the calculated increase in PGD activity.

No change in the ICD activity of normal serum was observed after storage at room temperature for 5 hours, or for many days at 4°. Serum PGD was more labile than ICD, approximately 50% of the activity was lost after storage at 4° for 4 days. Dialysis of serum against 0.15 M NaCl at 4° for 12 hours did not affect serum ICD.

TABLE I. Activity of ICD and PGD in Sera of Apparently Healthy Individuals.

Enzyme	Group	Age range (yr)	No. of subjects	Enzyme activity (mμmoles TPNH formed/1 ml of serum/hr at 25°C)
ICD	Males	21-49	22	110 ± 49* (55-224)
"	Females	21-49	22	109 ± 56 (47-264)
"	Term pregnancy	20-44	5	116 ± 36 (89-170)
"	Newborn infants		5	305 ± 91 (179-475)
PGD	Males	21-33	16	137 ± 36 (80-188)
"	Females	21-45	10	137 ± 60 (40-238)
"	Term pregnancy	16-25	3	178 (115,195,224)

* ± stand. dev.

Figures in parentheses represent range of values observed.

Stroma-free hemolyzates exhibited approximately 80 times the ICD and 450 times the PGD activity of the corresponding normal sera. Thus, reliable values for the activity of these enzymes can be obtained only with sera which show no visible trace of hemolysis. A number of experiments showed that contact of serum with erythrocytes for as long as 5 hours at room temperature did not change either ICD or PGD activity provided that no hemolysis had taken place.

ICD and PGD in the serum of apparently healthy individuals. The levels of ICD and PGD in the sera of apparently healthy individuals are summarized in Table I. It will be seen that the activity of both enzymes is of the same order of magnitude, *viz.* in the neighborhood of 50 to 250 mμmoles of TPNH formed per 1 ml of serum per hour at 25°. The statement in a preliminary communication(16) that the activity of these serum enzymes is one thousand times that depicted in Table I is due to a misprint. In adult subjects, no difference in the activity of either enzyme was found between the sera of males and females. Moreover, neither ICD nor PGD activity could be correlated with age, race, or total serum protein concentration. The sera of term pregnancies possessed activities for both enzymes which fell well within the normal range for adult females. However, sera from the cord blood of newborn infants showed somewhat elevated ICD levels. Repeated determinations of ICD and PGD in the serum of adults showed that the activity of these enzymes remained remarkably constant in a given individual.

Discussion. Although the presence of PGD in mammalian erythrocytes has been known for more than 20 years(17), the present findings make it clear that this enzyme and also ICD are true components of serum as well as of erythrocytes. The blood serum of normal individuals does not appear to contain all known TPN-linked enzymes. Unpublished experiments by us have shown that glucose-6-phosphate dehydrogenase activity of human serum is about the same as that of ICD and PGD. However, we have been unable to detect in the same samples any "malic" enzyme (7) or glutamic dehydrogenase which react with TPN.

When expressed in terms of moles of substrate transformed per 1 ml of serum per hour, the ICD and PGD levels found in normal individuals do not differ by a factor greater than 3 from the activities reported for the following serum enzymes: aldolase(10), glutamic-oxalacetic and glutamic-pyruvic transaminases(18), whereas the levels of lactic dehydrogenase(2,3) phosphohexoisomerase (11) and 5-phosphoriboseisomerase(19) are greater by orders of magnitude.

The vigorous ICD activity of human erythrocytes is in accordance with the observation of Rubinstein *et al.*(20) that certain enzymes of the tricarboxylic acid cycle including ICD are retained in the mature mammalian red cell, despite the fact that the respiratory metabolism of these cells is very feeble in the absence of unnatural electron carriers(21).

The factors which determine the extreme elevations in the activity of serum ICD in certain diseases of the liver(16) are under investigation.

Summary. Methods for determination of isocitric and 6-phosphogluconic dehydrogenases in human blood serum are described. The properties of these enzymes, and factors affecting their activity in the blood sera of

normal human adults are discussed.

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Mast Cell Changes in Animals Exposed to Cold. (23440)

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A marked increase in number of mast cells of the lungs and intestine of hedgehogs during hibernation was recently reported(1). Since mast cells are known to be an abundant source of heparin, the authors concluded that the increase of these cells was an important factor in preventing blood coagulation and thrombosis and in decreasing the work of the heart during hibernation. More recently a great deal of evidence has accumulated which strongly favors the opinion that mast cells are also an important source of histamine(2,3,4, 5).

We planned this study on mast cell changes in the cold for the following reasons. First, knowing that corticosteroids cause a degranulation of mast cells(6), it was suspected that during a long exposure to cold, when definite changes in the adrenal activity have been shown to occur(7), some changes in number or structure of these cells would take place. Secondly, measurements of histamine content in laboratory animals(8) and indirect evidence in humans(9) have shown that local application of a cold stress releases histamine from the stimulated tissues. Finally, the cold induced vasodilation normally observed after exposure to low temperatures, was associated with histamine release from the skin(9,10).

Methods. Experiments were conducted at 2 ± 1°C and at 6 ± 1°C; work to be reported elsewhere has shown that histological changes induced by cold are different at these 2 temperatures. White male rats, weighing approximately 300 g were used. In Exp. A, where animals were exposed to 2 ± 1°C, three groups of 12 rats were used. The first group, kept at 23°C, was the control group, whereas the second and third were respectively exposed to cold for 2 and 4 weeks. In Exp. B the same procedure was used with the exception of the temperature which was 6 ± 1°C instead of 2 ± 1°C.

The following procedure was used for all

TABLE I. Variations in Number of Mast Cells in Mesentery and in Skin of the Ear and Abdomen of Rats Maintained at 2 and 6°C for Periods of 2 and 4 Weeks.

Tissue	Normal (No./3 mm²)	6°C		2°C	
		2 wk	4 wk	2 wk	4 wk
Skin (ear)	92 ± 18*	70 ± 18	64 ± 12	36 ± 18	26 ± 22
Skin (abdomen)	252 ± 60	402 ± 36	—	372 ± 18	—
Mesentery (intervascular)	300 ± 86	192 ± 91	260 ± 89	140 ± 62	294 ± 100
Mesentery (perivascular)	1†	3	3	4	3

* Stand. dev. † Estimated intensity (0 to 5 scale).

animals to prepare tissues for mast cell examination. Light ether anesthesia was induced prior to excising 5 mm² sample of the ear, top of hind paw and mid abdomen. Two 25 mm² portions of the mesentery were also obtained. The mesentery samples which were taken as close as possible to the stomach, were mounted on 2 glass concentric rings having inside diameters of 25 and 30 mm. These samples were immediately fixed in alcohol 100% for 5 minutes, then in alcohol 80% and 60% for 2 minutes, washed in water for 2 minutes, stained with Pugh solution* for 3 minutes, washed for 2 minutes and taken up to alcohol 100%. After quick examination under a dissecting microscope, the specimens were mounted permanently on slides with "Permount." The skin specimens after being fixed with absolute methyl alcohol for 24 hours were also stained with toluidine blue. Alcohol was preferred to formalin as a fixative because the mast cell granules are water soluble. In the mesentery a whole 3 mm² field was counted whereas in the skin 6 fields of 0.5 mm² were examined.

Results. Mesentery. Table I shows a large increase in concentration of mast cells in the perivascular region and a significant decrease in the intervascular areas after 2 weeks in the cold at both 2 and 6°C. (Typical preparations are shown in Fig. 1 and 2). The difference in the cell counts in the intervascular region of the mesentery between the animals exposed for 2 weeks at 2°C and those exposed for the same period at 6°C, (Table I) is not statistically significant. After 4 weeks in the cold the number of mast cells in the perivascular areas returns almost to the control value.

Skin. Table I shows a significant increase in number of mast cells in the skin of the abdomen after 2- and 4-week exposures at both 2 and 6°C. However, in the skin of the ear there is a decrease in the number of these cells, a decrease significantly greater at 2°C than at 6°C under all conditions tested. In contrast, the changes after 2 weeks in the cold in the skin of the abdomen and in the mesentery were essentially the same at 2°C and at 6°C.

Discussion. Numerous studies have shown

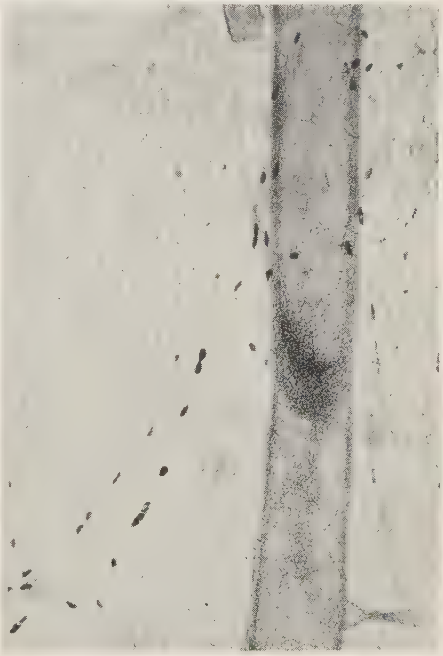

FIG. 1. Mast cells in perivascular region of mesentery of normal animals (× 80). Stained with toluidine blue.

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* Pugh solution is made by dissolving 0.4 g of toluidine blue in 4 ml ethyl alcohol, 86 ml distilled water and 10 ml glacial acetic acid.

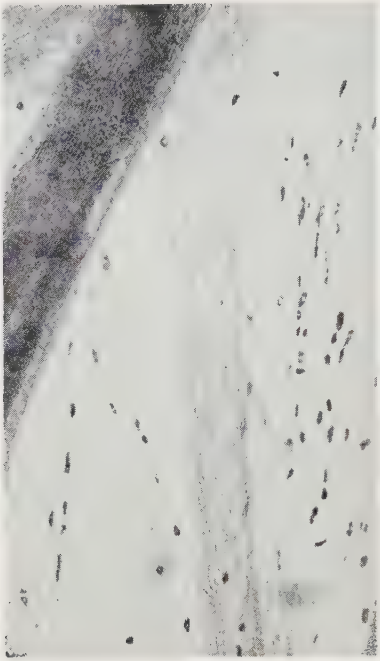

FIG. 2. Mast cells in perivascular region of mesentery of animals exposed to cold for 4 wk at 6°C (× 80). Stained with toluidine blue. A large increase in No. of mast cells is observed. This increase is also noted after 2 wk at 6°C and after 2 and 4 wk at 2°C.

that systemic acclimatization to low temperatures is associated with ability to maintain a sustained increase in heat production. This modification is made possible by the relatively slow process of gradually increased endocrine activity. On the other hand fewer studies have been made on local adaptation or on the interrelationships between local and systemic acclimatization. We have recently described a local and systemic adaptation to a locally applied cold stimulus in humans(9). Generally a larger number of studies are being carried out on systemic adaptation in laboratory animals than in humans, whereas the reverse is true for local adaptation studies. The present study was designed to distinguish between the local and systemic effect of cold on mast cells. Indeed changes in the number and in the structure of these cells in the extremities and ears, which are directly exposed to cold and chilled to temperatures nearing those of the environment, could be different from the changes taking place in parts of the body which are covered by hair or in internal organs such as the mesentery. The systemic effect of cold on number of mast cells results in an increase as evidenced by changes in the perivascular areas of the mesentery and in skin of the abdomen. Furthermore, this increase is the same whether exposure temperature was 2 or 6°C. This last effect was expected to some extent since the systemic response to these 2 environmental temperatures, which are so close to one another, could not be too dissimilar. On the other hand the local effect of cold on number of mast cells is contrary to the systemic effect since we have observed a decrease of these cells in the skin of the ear. Of additional interest is the fact that this decrease is much larger at 2° than it is at 6°C. Work being completed at our laboratories shows a marked difference in the histological response to these two temperatures. For instance, the edema is much more pronounced at 2° than it is at 6°C.

The question of primary importance is to find out if the increase in mast cells of the perivascular region of the mesentery and of the skin of the abdomen is an indication of increased production or decreased utilization of the substances known to be present in the granules of mast cells. Recently Fawcett(4) described an "active amoeboid migration" of mast cells from the perivascular region of the mesentery into the avascular areas, while Mergenthaler and Paff(11) gave some evidence for actual "shelling out" of these cells from the mesentery. Consequently, in the present study the increase in the perivascular mast cells may be interpreted as an increased production whereas the decrease in the avascular region of the mesentery would indicate an increased utilization. The final result of these changes should be a larger amount of heparin or histamine in the peritoneal cavity or probably even better in the circulation. This same suggestion was made by Harma, *et al.*(1) who also found an increase in the number of mast cells in the lungs and intestines of hedgehogs in hibernation. The final proof would of course be obtained by determination in the blood of substances known to result from mast cell degranulation, such as histamine or heparin.

The cause for these local and systemic reactions is incompletely understood although different studies bring sufficient evidence to support some speculative views on this question. Pellerat and Murat(8) have shown that chilling human skin causes a decrease of histamine in the skin and an increase in the blood. We obtained indirect evident to this effect in humans(9). Consequently, the decrease in number of mast cells in the ear of rat exposed to cold is a local effect of this stress. The reason for the systemic changes observed is not obvious. However, the fact that degranulation of the mast cells in the intervascular region of the mesentery observed after 2 weeks in the cold is not evidenced at 4 weeks could indicate that these systemic changes are controlled by some mechanism which is less intense after 4 weeks in the cold than after 2 weeks. The adrenal cortex secretion in the cold has been shown by Heroux and Hart to follow this general pattern(7). These authors observed greater activity of the adrenal cortex at 2 weeks than at 4 weeks in animals exposed to cold.

These changes in mast cells in the cold will be understood only when their significance can be estimated in terms of our present knowledge of local and systemic acclimatization to cold.

Summary. In the mesentery the number of mast cells increased in the perivascular region after exposing the animals for 2 and 4 weeks at 6°C; in the intervascular region the number decreased after 2 weeks but returned to the initial value after 4 weeks. The same changes were observed in animals exposed at 2°C. In the skin of the abdomen an increase was observed at both temperatures after 2 weeks. In the skin of the ear, which is much more exposed to the environmental temperature than the abdomen, a significant drop was observed after 2- and 4-week exposures to 2 and 6°C. However, the decrease was significantly larger at 2°C than at 6°C. The increases in number of mast cells in abdominal skin and the changes in the mesentery are considered to be systemic effects of cold; the decreases in the skin of the ear are considered to be local effects.

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Effect of Stage of Gestation on Succinoxidase Activity of Fetal and Maternal Swine Hearts.*† (23441)

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Sippel(4) has demonstrated that heart function and succinoxidase activity are closely related in rat and chick ventricles during embryonic development. Cooper(1) found that succinic dehydrogenase in the 20- and 21-day-old fetal rat ventricle was approximately 30% of that of the adult rat. The present study was made to determine (a) the rate at which swine develop this active enzyme system in the heart of the embryo, and (b) the influence of different stages of gestation on the maternal heart. In addition, two dietary levels of manganese and position of the embryos in the uteri were observed for their influence in the above tests.

Experimental. Eighteen Duroc gilts weighing 277 ± 20 lb. were divided into 2 equal lots. They were fed a basal ration containing 10% dried skim milk, 10% tankage (meat and bone), 76.5% ground yellow corn, 3% citrus meal and 0.5% iodized salt. The following components were also added per ton: 4 g riboflavin, 8 g pantothenic acid, 18 g niacin, 20 g choline-Cl, 0.12 g folic acid, 3.6×10^6 U.S.P. units of vit. A, and 0.45×10^6 units of vit. D. This ration contained 7 p.p.m. of manganese by analysis. One lot of 9 gilts received this ration; another lot received the same components but in addition sufficient $MnSO_4 \cdot 7H_2O$ to increase the manganese to 68 p.p.m. Since the level of manganese in the ration had no statistically significant influence on the succinoxidase activity of the heart, or on fetal growth, or maternal vigor, the data for this treatment are not separated in this report. The sows averaged 50 days on the rations before they began their gestation periods, of 112 days. The fetal heart begins to function at about 25 days of age.

Six pregnant sows were sacrificed at 60, 80, and 110 days of gestation, respectively. Determinations were immediately made of the succinoxidase activity of the fetal ventricles, and the left ventricles of the sows. Hearts of fetuses from the extremes and middle areas of the uteri were analyzed, but position was not found to influence enzyme activity; hence, individual values for hearts of fetuses in the various positions are not presented. Succinoxidase activity was determined by the method of Schneider and Potter(2). Nitrogen was determined in the homogenates by the Kjeldahl method. The succinoxidase activity was calculated as ml of oxygen uptake per mg of N per hour, and as ml of oxygen uptake per gram of wet weight per hour.

Results. The data obtained for the succinoxidase activity for the fetal and maternal hearts are presented in Fig. 1. The activity is expressed as ml of oxygen uptake per gram of wet weight, and per 10 mg of nitrogen per hour. Statistical evaluation was made of the data according to Snedecor(5). The increase in succinoxidase activity with increase in age of the fetuses was significant ($P<0.01$) on both the wet weight and the nitrogen basis. The increase in activity was accelerated somewhat between 80 and 110 days over that between 60 and 80 days of gestation. This increase is not as marked as the 2 increases that Sippel(4) observed during 2 stages in the embryonic development of the rat; but it does show that in swine the greater function of the fetal heart near the end of gestation is accompanied by an increase in the succinoxidase activity. This was true on both the wet weight and the per unit of nitrogen basis.

The succinoxidase activity of the maternal hearts was approximately 6 times greater than that of the fetuses at 60 and 80 days, and about 4 times greater at 110 days of gestation, on the wet weight basis. On the basis of activity per mg of nitrogen the maternal hearts

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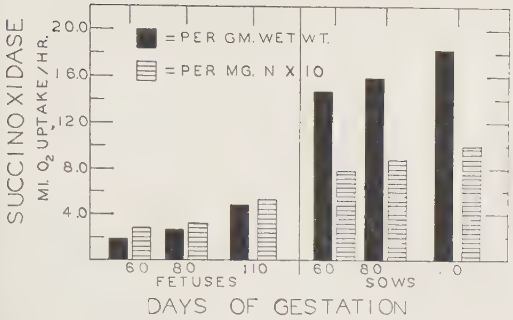

FIG. 1. Effect of 3 stages of gestation in swine on the succinoxidase activity of fetal and maternal heart ventricles. Significance: Fetuses ($P < 0.01$). Sows ($P < 0.05$) for data on wet wt basis; approaches significance on the nitrogen basis.

had only about 3 times as much activity at 60 and 80 days and approximately twice as much at 110 days of gestation as the fetal hearts. The greater activity of the maternal hearts on the wet weight basis checks quite closely with the relative values of the adult and fetal rat ventricles observed by Cooper(1). The "efficiency" of the 110-day swine fetal ventricle compared to the corresponding maternal ventricle is about doubled when the activity is expressed on the per unit weight of nitrogen basis instead of per unit of wet weight *i.e.* a ratio of 1:2 is obtained instead of 1:4. The increased succinoxidase activity of the ventricles of the sows with increasing stage of gestation was significant ($P<0.05$) on the fresh weight basis, and approached significance on the basis of activity per mg of nitrogen.

Shirley, *et al.*(3) found that gilts fed a corn-soybean-tankage-alfalfa meal ration *ad libitum* had succinoxidase activity of 21 to 24 ml O_2 uptake/g fresh wt/hr in the left ventricles at 3 and 25 days of gestation which is somewhat greater than that found in the heavier sows at 60, 80 and 110 days of gestation in the present study. When the above gilts were limited to approximately one-half the normal dietary energy intake, succinoxidase activity of 17.6 to 18.5 ml O_2 uptake/g fresh wt/hr was observed, which is in the range of that of the sows of the present study. These data indicate that advanced gestation results in a decrease of succinoxidase activity in the maternal heart to the extent observed when the sows were fed a low energy ration. Essentially no difference in enzyme activity occurred between 3 and 25 days of gestation in the gilts. The increase in activity observed in the present study with the sows at 60, 80, and 110 days of gestation, indicates that as gestation approaches termination, the physiological demands result in a greater capacity of the energy producing succinoxidase system of the maternal heart.

Summary. A significant increase in the succinoxidase activity of fetal swine ventricles was found to occur during the latter half of gestation, on both the wet weight and per unit of nitrogen basis. As gestation approached termination, an increase in activity occurred in the left ventricles of the maternal heart. These observations show that in large animals the fetal heart increases in succinoxidase activity with increased function, as previously demonstrated in the rat and chick; and that the later stages of gestation make increased requirements of the heart as indicated by greater maternal heart enzymatic activity. Two dietary levels of Mn and position of the fetuses in the uteri had no influence on the succinoxidase activity.

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Immunological Relationship Between Measles and Distemper Viruses.* (23442)

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The following experiments are being reported because of previous studies pointing to a definite pathological and immunological relationship between canine distemper and a human respiratory disease(1,2). Pinkerton, *et al.*(3) suggested a possible biological relationship between measles and distemper principally on the basis of pathological data. Both measles and distemper viruses produce characteristic cytoplasmic and intranuclear inclusion bodies in epithelial tissues, and also have the property of causing multinucleated giant cells(4). As yet, however, no evidence has been submitted of an immunological relationship between these two agents. The finding that measles or rubeola virus (Edmonston strain)(5) could be established in HeLa cell cultures(6,4) as well as KB cells(7) and Hep-2 cells(6), suggested a method of exploring the problem. The experiments reported here indicate that measles propagated in HeLa cell cultures could be neutralized by specific canine distemper (Carre's virus) antiserum obtained from immunized ferrets, and that distemper virus infection in animals could be neutralized by measles antiserum. These immunological cross-reactions suggest that common antigenic components are shared by the viruses of measles and distemper.

Materials and method. Methods of study. Three principal methods were employed to demonstrate the immunological relationship between measles and distemper viruses: 1. In tissue culture, measles virus was neutralized by specific distemper antiserum. The antiserum was prepared in ferrets by inoculation with either egg-adapted or mouse-adapted distemper virus. 2. In ferret protection studies, ferrets were immunized with measles virus and subsequently challenged with virulent distemper virus. 3. Mouse-adapted distemper virus was neutralized by human measles convalescent sera and by sera from ferrets that were immunized with measles virus.

Viruses. The Edmonston strain of the measles virus was successfully propagated and maintained in HeLa cell cultures. Cytopathic changes consisted of characteristic multinucleated giant cells with cytoplasmic and intranuclear inclusion bodies (Fig. 1). The identity of the virus propagated in HeLa cells was established by neutralization and complement-fixation tests (Table I). The neutralization test was carried out by mixing 200 TCD_{50} of the viral tissue culture supernatant with dilutions of acute and convalescent serum from patients with typical measles. These mixtures were incubated at 37°C for ½ hour and at 4°C for an additional ½ hour before inoculation of HeLa cultures. Supernatant fluids from infected cultures were also employed as antigens for complement-fixation tests. These tests indicate that significant increases in neutralizing and complement-fixing antibodies occurred in the serum of the patients with clinically diagnosed measles.

The Lederle strain of avianized distemper virus(8) was maintained by successive serial passages on the chorioallantoic (CA) membrane of 8-9-day-old chick embryo. A 10% CA membrane suspension of 1 ml amount was injected intraperitoneally into ferrets, which subsequently survived a challenge of 100 MLD virulent ferret distemper virus.

The mouse-adapted distemper virus(9,10,11) was developed by successive serial passage in brain of suckling mice with egg-adapted distemper virus. The LD_{50} of this virus after 45 passages in suckling mice is about $10^{-3.5}$ with an incubation period of 4-5 days. Ferrets were inoculated intraperitoneally with 1 ml of 20% brain suspension from the 54th passage material. These ferrets subsequently survived a challenge of 100

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TABLE I. Measles Neutralization and Complement Fixation Tests with Human Sera.

		Serum dilution	
Sample	Serum	Neutralization titer	CF titer
1	Acute	<1/10	<1/ 16
	Convalescent	1/80	1/128
2	Acute	<1/10	<1/ 16
	Convalescent	1/40	1/ 64

MLD virulent ferret distemper virus.

Neutralization of measles virus. The HeLa cell cultures were grown and maintained in medium containing 60% yeast extract medium(12), 20% Scherer's maintenance solution(13), 10% calf serum and 10% brain heart infusion broth. The medium was changed every 3-4 days. In the neutralization studies measles virus representing 200 TCD_{50} was mixed with equal amounts of diluted test sera. Mixtures were incubated at 37°C for ½ hour and placed in the refrigerator for an additional ½ hour. All test sera were inactivated at 56°C for ½ hour prior to use. A standard inoculum of 0.2 ml was added to the HeLa cell culture tubes and observed daily for 21 days. Tests were also carried out by mixing diluted serum with serial virus dilutions and incubating similarly before inoculation of HeLa cell cultures.

Ferret protection studies. In 3 separate experiments ferrets were immunized with measles virus and subsequently challenged with virulent distemper virus. In the first experiment, 8 ferrets were given 2 intramuscular injections of HeLa passage measles virus 2 weeks apart and challenged 2 weeks later with approximately 100 MLD virulent ferret distemper virus. In the second experiment, 5 ferrets were given 4 intramuscular injections of measles virus at approximately 3 week intervals. These animals were challenged 3 weeks after the final injection with approximately 50 MLD virulent ferret distemper virus. In the third experiment, 7 ferrets were inoculated intramuscularly with 1 ml of a mixture of measles virus and adjuvant(14) prepared as follows: 1 part arlacel A, 9 parts bayol F and 10 parts HeLa virus suspension. Approximately 1 month and 2 months later, the animals were again inoculated intramuscularly with 1 ml of measles virus without adjuvant. These animals were subsequently challenged with 100 MLD virulent ferret distemper virus. In each experiment, control ferrets included animals receiving no previous treatment and also animals receiving normal tissue culture fluid instead of measles virus suspensions.

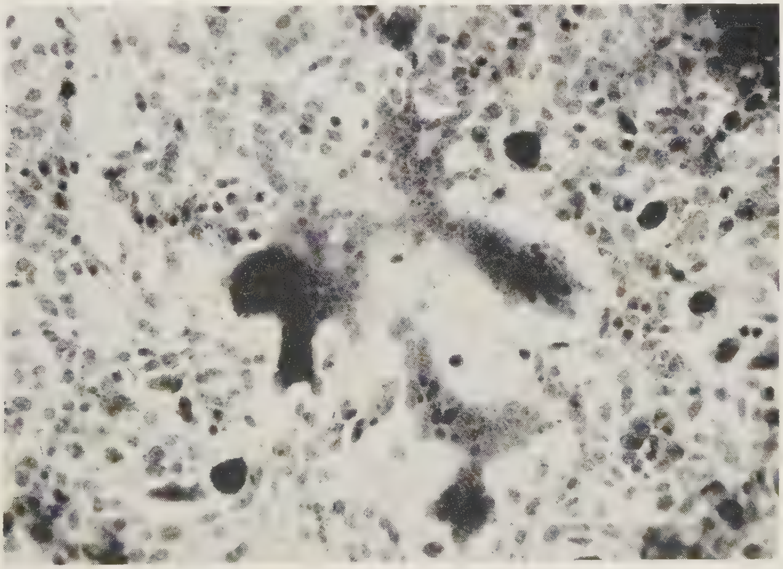

FIG. 1. Photomicrograph of HeLa cell tissue culture showing typical cytopathology with multinucleated giant cells produced by measles virus. Photographed by Timothy Dodge.

TABLE II. Effect of Addition of Fresh or Heated Normal Guinea Pig Serum on Neutralizing Capacity.

Antiserum samples	Neutralizing antiserum diluted with BSS*	 Inactivated N.G.P. serum	 Fresh N.G.P. serum
1	1/ 2	1/ 2	1/ 16
2	1/16	1/16	1/ 64
3	1/32	—	1/128

* BSS—Hank's Balanced Salt Solution.

Neutralization of distemper virus. Equal amounts of the mouse-adapted distemper virus and serum were incubated at 37°C for ½ hour and 4°C for additional ½ hour before injecting 0.01 ml intracerebrally into suckling mice. Neutralization was determined by survival of the animals. Tests were also carried out by mixing serum with serial virus dilutions and incubating similarly before inoculation. All test sera were inactivated at 56°C for ½ hour before being employed for neutralization studies.

Results. The neutralization data show that sera from ferrets immunized with mouse-adapted and egg-adapted distemper virus inhibited cytopathogenesis due to measles virus. Final serum titers of ½, ¼, ⅛ and rarely greater than 1/16 were recorded. These sera were drawn 4 weeks following the severe distemper challenge. In no case did preimmunization or normal serum show any inhibition of measles virus. A representative neutralization test which was carried out by mixing 1/5 dilution of ferret serum with serial virus dilutions shows that 2.5 logs of measles virus were neutralized by the serum from a ferret immunized with mouse-adapted distemper virus.

When complement as present in fresh normal guinea serum was added to the ferret sera, the enhancement of neutralizing properties was demonstrated; when normal guinea pig serum was inactivated by heating for ½ hour at 56°C no enhancement was evident (Table II).

Animal studies employing ferrets have been partially successful in confirming the findings in tissue culture. In the following 3 experiments ferrets were immunized with measles virus and subsequently challenged with virulent ferret distemper virus. Evidence of protection was revealed by prolonged incubation periods and modified illness with survivals. These results are summarized in Fig. 2. Among the 20 experimental animals, 8 survived and 12 died, whereas only 1 animal survived the challenge among 17 controls. Serum from the 1 surviving control ferret which received only distemper virus showed a rise in measles antibody from 0 to ⅛.

Representative results of neutralization of mouse-adapted distemper virus by specific measles antiserum prepared in ferrets are presented in Table III. Employing 300 LD_{50} of mouse-adapted distemper virus and undiluted measles antiserum obtained from ferrets in the preceding experiments, neutralization studies were carried out by inoculation of the mixture intracerebrally into 1- and 2-day-old mice. These results indicate that specific measles antiserum completely protected the mice against the lethal effects of the virus, whereas, preimmunization serum and control ferret serum had no inhibitory effect. Dilution studies revealed complete protection at ¼. Neutralization of the virus was also evident when mouse-adapted and egg-adapted distemper antisera were employed in the study. Similarly, when neutralization tests were carried out by mixing undiluted ferret sera with serial virus dilutions, 3.5 logs of distemper virus were neutralized by the serum from a ferret immunized with HeLa passage measles virus.

Table IV illustrates the fact that mouse-adapted distemper virus is neutralized by serum antibodies developing during the course of clinically diagnosed measles. Employing 100 LD_{50} of mouse-adapted distemper virus and sera obtained from acute and convales-

TABLE III. Neutralization of Mouse Adapted Distemper Virus with Ferret Measles Antiserum.

Serum samples	Suckling mice, # dead/# inj.
Normal ferret (pre-immunization)	8/8
Ferret immunized with measles	0/7
Normal ferret (pre-immunization)	4/4
Ferret immunized with measles	0/9
Ferret immunized with:	
Control culture	7/7
Avian-distemper	0/6
Mouse-distemper	0/7

TABLE IV. Neutralization of Mouse-Adapted Distemper Virus by Human Convalescent Measles Sera.

Sample	Serum	Neutralizing serum dilution*
1	Acute	<1/ 4
	Convalescent	1/64
2	Acute	<1/ 4
	Convalescent	1/43

* Expressed as dilution of serum which protected 50% of suckling mice from death.

cent stages from 2 patients with measles, neutralization tests were carried out in suckling mice. These results indicate that significant increases in distemper neutralizing substances occurred in the serum of both patients. These two patients also had significant increases in measles antibody in complement-fixation and neutralization tests.

Discussion. It would appear that measles and distemper are as yet 2 distinct entities caused by well recognized respiratory viruses in their respective hosts, man and dog. Although both of these diseases occur in epidemics with similar incubation periods and are characterized by similar symptom patterns including a rash, there exist many unsolved problems with respect to the antigenic properties of the agents. The measles virus is readily adaptable to many different tissue culture preparations whereas the distemper virus, although adapted to certain animals, mice and the chick embryo, has been found difficult to produce cytopathogenesis in tissue culture. The pathologic similarities have been emphasized in previous reports(3,4), including the striking formation of multinucleated giant cells, and typical cytoplasmic and intranuclear inclusion bodies produced by both viruses in epithelial tissues.

Cross neutralization studies reported in this paper have demonstrated the development of neutralizing factors in the serum of animals and man by both measles and distemper virus.

It has been demonstrated(1,2) that human serum was capable of neutralizing distemper infection in chick embryos and in ferrets, and in this paper human serum from patients recovering from measles has been shown to protect mice completely from death caused by distemper virus. The acute or early specimen from these same patients has failed to protect the mice from dying. Recently both Karzon(15) and Carlström(10) demonstrated distemper antibodies in human beings. In both studies, the serum dilutions employed were 1/5. It is possible that cross-neutralization between measles and distemper might be responsible for the data presented.

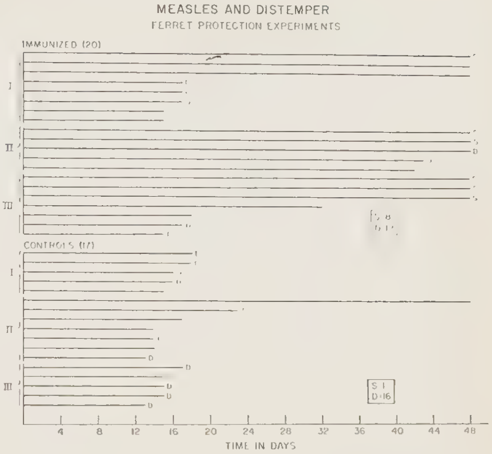

FIG. 2. Summary of 3 experiments showing results of challenge with distemper virus of ferrets immunized with measles virus; duration of illness (D) and survivals (S) is charted for comparison with the controls.

Summary. In tissue culture studies, the Edmonston strain of measles virus was neutralized by distemper antiserum prepared in ferrets with the egg-adapted and mouse-adapted strains of distemper virus. All normal ferret sera failed to show any neutralization of measles virus. In animal studies, ferrets immunized with measles virus and subsequently challenged with virulent distemper virus showed some evidence of protection as revealed by prolonged incubation periods, modified illnesses and survivals. The mouse-adapted distemper virus was completely neutralized by measles antiserum prepared in ferrets, whereas, normal serum failed to show any neutralization. Mouse-adapted distemper virus was also neutralized by human measles convalescent sera. These results suggest that common antigenic components are shared by the viruses of measles and distemper.

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Atherosclerotic Response of "Aggressive" and "Passive" Groups of Chickens To a Cholesterol Enriched Diet.* (23443)

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The possible effect of various emotional factors upon development of hypercholesteremia and atherosclerosis in laboratory animals also given a cholesterol enriched diet rarely has been studied. It was thought advisable to attempt such a study in respect to one emotional factor, namely, degree of aggressiveness as observed in young male chickens. This species was considered for such a study because of the well known pecking order (which might be considered a form of aggression) often observed in a flock of chickens(1,2) and susceptibility of chickens to dietary induced atherosclerosis(3).

Methods. In order to study male chickens ostensibly exhibiting either "aggressiveness" or "passiveness", a total flock of approximately 2500 male chickens (Avg. age: 6 weeks) was observed carefully by one of us (C.A.) for a number of hours both before, during and after feeding periods. A preliminary selection of 100 most "aggressive" chickens and 100 most "passive" chickens then was made. The characteristics of aggression were considered to be (1) insistence and speed of obtaining proffered feed, (2) over-all general physical activity and (3) agitation and belligerent resistance when handled. Passive characteristics were considered to be the converse of the preceding traits.

Following this preliminary selection, the 2 groups were housed in respective coops and were observed daily for 2 weeks during which time, the 30 most "aggressive" and 30 most "passive" chickens were selected from each. After this selection, the chickens were tagged with leg bands. Each group then was housed in 2 coops (approximately 5 feet square). The "aggressive" chickens were removed from their coops every 2 weeks, intermingled and then replaced in the coops. This was done in order to prevent a few from gaining group ascendancy. The "passive" chickens in the other 2 coops were treated similarly.

The animals were fed twice daily with a standard poultry mash (Albers Pullet Grower) enriched with cottonseed oil (5%) and cholesterol (2%). Routinely, the "passive" chickens were fed first and time allowed for feeding was one hour. At the end of this

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* Aided by a grant from the Alameda Heart Assn.

TABLE I. Atherosclerotic Response of "Aggressive" and "Passive" Chickens to Cholesterol Enriched Diet.

	"Aggressive group"			"Passive group"		
Final: Avg wt (oz)	84	± .94†	(80-91) ‡	77	± 2.98†	(59-84) ‡
Comb wt (g)	29.4	± 1.26	(22.6-35.6)	21.6	± 1.98	(10-26.9)
Testes wt (g)	17.8	± 1.2	(12.1-24.8)	13.2	± 1.7	(8-22)
Avg plasma cholesterol (mg/100 ml)	348	± 40.1	(180-599)	525	± 76.9	(166-905)
Avg aorta cholesterol (mg/100 g)	1264	±226	(929-3709)	1957	±294	(1035-3150)
Degree of coronary atherosclerosis (%)*	45	± 8.5	(1-88)	47	± 7	(14-80)

* This has been calculated as the ratio of No. of vessels exhibiting Sudanophilic staining to total No. of arteries and arterioles observed.

† ± stand. error of mean.

‡ Range.

period, the amount of food ingested was calculated, then this exact amount was proffered to the herd of "aggressive" chickens. The average amount of mash ingested was approximately 3.5 lbs per feeding. It was of interest that the "aggressive" fowls regularly consumed their portion of food approximately 15 minutes more rapidly than the "passive" animals. Despite this arbitrary equivalence of food intake, it was observed at the end of the seventh week of feeding that the "aggressive" group exhibited a greater gain in weight. Therefore, an additional pound of food per feeding was given to the "passive" group for the remaining 8 weeks.

Blood samples were drawn from the axillary vein and the serum was analyzed for cholesterol on the first, 8th and 15th week of the experiment, using the method of Zak *et al.*(4). The average plasma cholesterol of each group was computed by averaging the 3 cholesterol values thus obtained. Weights were recorded on the first, 4th, 8th and 15th weeks.

During the course of the experiment some of the "aggressive" chickens were observed to exhibit a more passive type of behavior and conversely, some of the "passive" group later exhibited aggressive characteristics. At the conclusion of the experiment then, 12 "aggressive" and 9 "passive" chickens were obtained that had exhibited a consistent behavior pattern from the beginning.

After the 15th week the chickens were killed by bleeding. The testes, upper comb, heart and a segment (4 cm) of the arch and thoracic segments of the aorta were removed from each chicken. The weight of the testes and combs were recorded. The aortic segments were analyzed for cholesterol by previously described methods(5). Three transverse sections were obtained of the entire left ventricle (base, middle and apex) and were stained with Sudan IV. The coronary vasculature (arteries and arterioles) in these sections then were examined microscopically for Sudanophilic staining. A ratio of the number of vessels so stained to the total number of vessels observed (approximately 80 in each heart) was obtained.

Results. As might be expected, average weights of the 12 "aggressive" chickens and of the 9 "passive" chickens were approximately the same (Table I). It was of interest that secondary sex characteristics of the aggressive group (as indicated by weights of comb and testes) appeared to be more pronounced.

Rather surprisingly, average plasma cholesterol of the "passive" group was approximately 34% higher than that of the aggressive group (Table I). Similarly cholesterol content of aortic segments of the "passive" group was also higher (35%) than that of the "aggressive" group. It is, however, somewhat questionable whether these differences are significant in view of the relatively small number of animals and the wide range of individual values of both plasma and aortic cholesterol contents.

Despite the above possible differences in development of secondary sex characteristics and cholesterol contents of plasma and aorta respectively, no difference was observed (Table I) in extent or degree of coronary atherosclerosis as indicated by the ratio of affected to total number of normal vessels seen.

Summary. A group of male chickens ex-

hibiting "aggressive" and another group exhibiting "passive" behavior were studied during and after a period of high cholesterol and fat feeding. Although the "aggressive" groups exhibited more pronounced evidences of androgen activity, their plasma and aortic cholesterol contents were perhaps less than those of the "passive" group. The extent and degree of coronary atherosclerosis was the same in the two groups.

We wish to express our thanks for the technical assistance of Clarence Omoto and Warren Hayashi.

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Altitude Stress: Its Effect on Tissue Citrate and Salmonellosis in Mice.* (23444)

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Mice exposed to simulated altitude of 20,000 ft for 3 weeks or for 6 weeks show a uniform percentage decrease in tissue citric acid content and in their susceptibility to salmonellosis(2). Attempts to verify these findings in guinea pigs native to the Andean altiplano compared to those native to the coastal plain of Peru were not convincing for tissue citrate because of the wide range of individual values found within groups(3). The present communication presents results of studies on mice subjected to simulated altitude for a period of 3 to 4 months along with additional measurements designed to elucidate possible causes for the observed changes in tissue citrate.

Methods. Mice were exposed to simulated altitude in decompression chambers according to the schedule and technic previously described(1) with one important modification. Rather than control pressure within the chamber by a relay, an adjustable needle valve was substituted. The pressure of a desired altitude could be maintained during continuous operation of the pump with a variation in 24 hrs of approximately 3 cm of mercury (± 1,000 ft). Daily, the chambers were brought to atmospheric pressure while the cages were cleaned and fresh food and water were added. After about 15 to 20 minutes the animals were again at simulated altitude. The method of Ettinger *et al.*(4) was used for citric acid assays on blood, liver, spleen, kidney, duodenum, diaphragm, heart, and lung. Each analysis was done on the pooled tissues of 3 mice. Animals were killed by decapitation. The blood for assay was collected in a paraffined watch glass and transferred by tuberculin syringe to tungstic acid, the protein precipitant. The tissues were dissected quickly from each animal in 75 to 150 mg amounts and placed directly on dry ice. They were weighed in the frozen state, returned to the dry ice until all tissues were collected, and then they were homogenized in cold tungstic acid using a teflon in glass homogenizer. CF 1 female mice were used in all experiments. They were fed Purina dog chow *ad libitum* and water was available at all times.

Results. Citric acid in tissues of control mice. The mean values of citric acid (μg/g wet wt) for each of the tissues are given in column 2, Table I. These agree closely with

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TABLE I. Citric Acid Content of Tissues of Normal Mice, of Mice Exposed for 3-4 Months to a Simulated Altitude of 20,000 ± 1,000 Ft, of Mice Injected Intraperitoneally with 1 mg Cortisone Acetate Daily for 2 Weeks, and of Mice Starved for 17 Hours. Each value in the table is the mean ± stand. dev. of 8 separate determinations, each on the pooled tissues of 3 mice.

	Citric acid content (γ/g wet wt ± stand. dev.) of tissues from			
Tissue (1)	Control mice (2)	Mice kept at altitude 3-4 mo (3)	Mice given 1 mg cortisone acetate daily for 2 wk (4)	Mice starved 17 hr (5)
Blood	36 ± 6.6	23 ± 2.5	36 ± 3.2	130 ± 9.4
Diaphragm	86 ± 6.2	55 ± 8.1	79 ± 4.8	134 ± 10.4
Duodenum	84 ± 6.7	68 ± 6.9	91 ± 4.0	129 ± 10.6
Heart	55 ± 4.7	36 ± 3.2	54 ± 4.8	97 ± 6.7
Kidney	63 ± 5.3	41 ± 3.3	96 ± 6.1	72 ± 8.3
Liver	76 ± 6.6	58 ± 6.4	79 ± 5.6	87 ± 9.6
Lung	68 ± 4.3	52 ± 6.0	72 ± 5.6	148 ± 7.0
Spleen	92 ± 7.6	62 ± 5.6	79 ± 5.6	167 ± 13.2

values already published(2) except for a statistically significant difference in duodenum. The present value is lower by about 20 γ per g. The 2 experiments were carried out, however, more than 3 years apart.

Citric acid in tissues of mice adapted to altitude for 3-4 months. The results listed in column 3, Table I are about 30% (19% to 34%) lower than the corresponding control values. Duodenum shows the minimum percentage change. In each tissue the difference between means is significant at less than the 0.1% level as estimated by the rank order test of White(6). In no tissue was the largest value from altitude adapted mice as large as the smallest value for control mice. These data extend, therefore, the results previously reported for mice kept at altitude for shorter periods of acclimatization(2).

Citric acid in tissues of mice injected with cortisone acetate daily for 2 weeks. The possibility that the change in tissue citrate associated with acclimatization to altitude is mediated through a glycocorticoid from the adrenal was considered. As a means of testing this hypothesis, daily intraperitoneal injections of 1 mg of cortisone acetate (Nutritional Biochemicals) for 2 weeks into a group of normal mice preceded the assays for tissue citrate. The hormone was suspended in isotonic sodium chloride solution with a drop or two of Tween 80 as stabilizing agent. The desired amount of cortisone was contained in 0.5 ml of suspension. The number of injections was based on the previously observed fact that 2 weeks exposure to simulated altitude results in a statistically significant drop in citrate concentration in all tissues examined(2). The dosage level was arbitrary but an amount was desired that might reasonably be expected endogenously. The adrenals from several mice were weighed in the freshly frozen state. Total wet weights varied from about 8 to 11 mg per animal. To administer an amount of hormone equal to about 10% of total gland mass seemed, therefore, to be a more realistic dose than some that have been employed in mice for other experimental purposes as reported in the literature(5). The results of these tests are summarized in column 4, Table I.

It can be seen that the citric acid content of all tissues is not significantly different from that of control animals, with 2 exceptions. Kidney of cortisone-treated mice has more citric acid than in normal mice while spleen shows the reverse change. Under the conditions of the experiment, therefore, cortisone fails to produce tissue citrate changes of the general type found in animals acclimatized to simulated altitude.

Citric acid in tissues of mice starved for 17 hours. Blood sugar of mice exposed to simulated altitude for 3 weeks is approximately 30% lower than that of control animals (108 mg% *vs.* 149 mg%). Thus the altitude exposed animals have decreases in both blood sugar and tissue citrate of comparable magnitude. This suggests the possibility of a correlation between the two. An experiment was designed accordingly. Blood sugar was lowered by withdrawing food from otherwise

TABLE II. Survival Time of Mice Infected Intraperitoneally with 10^5 *Salmonella typhimurium*. The altitude mice were exposed to a simulated altitude of 20,000 ± 1,000 ft for 3-4 mo but were removed from the decompression chambers for the postinfection period.

Time post-infection (hr)	No. of mice surviving in group of	
	Altitude mice	Control mice
0	10	10
47	9	10
49	8	10
100	4	9
103	3	9
119	2	9
124	2	8
143	1	5
148	1	4
149	1	3
167	0	2
191		0
Mean survival time	103 ± 44	150 ± 27

normal animals for a period of 17 hrs. At the end of this time blood sugar was 75 ± 13 mg% and the tissue citrate was found to be that listed in column 5, Table I. In every case the citric acid is above the corresponding control value. The smallest percentage changes are seen in kidney and liver but these are significantly larger (P between 0.05 and 0.01) by rank order test(6). All other values, compared to controls, are significant at less than 0.001 probability.

Survival time of altitude acclimatized mice infected with Salmonella typhimurium. Table II presents the results obtained when mice exposed to simulated altitude for 3 to 4 months are infected intraperitoneally with 0.5 ml of a saline suspension of a 15-hour brain-heart infusion broth culture of *Sal. typhimurium* containing about 100,000 cells. The animals were kept at atmospheric pressures for the entire postinfection period. Their survival is compared with control mice similarly infected. The altitude mice are more susceptible as revealed by the lower survival time (P is approximately 0.01 by "t" test), even for this small sample.

Discussion. The earlier suggestion(2) that mice at altitude establish a lower steady state concentration of tissue citric acid seems to apply to animals maintained in the decompression chambers for periods up to 4 months. The extent to which these animals are truly acclimatized to their new environment is difficult to assess but they give every evidence of being in good physical condition. How much the observed shift in citric acid concentration is to be attributed to altitude *per se* or to the daily compression and decompression necessary for renewal of food and water remains to be determined. Even if it is the latter, it must be assumed that animals adapt to the downhill-uphill stresses within a period of about 3 weeks and daily repetitions for many additional weeks lead to no further metabolic shifts.

The biochemical change responsible for the altered tissue citrate remains unknown. The fact that injections of cortisone acetate failed to imitate the effects of altitude on tissue citric acid is not conclusive. The choice of dosage, of number of daily injections, and of adrenocorticosteroid were all arbitrary. Under other experimental conditions the results might have been more revealing. The findings with starved mice clearly prove, on the other hand, that no direct correlation exists between blood sugar level and tissue citric acid concentration.

The results of the infection experiment serve to confirm previous observations on similar groups of mice except that the altitude animals were exposed for only 3 weeks at the time of challenge(1,2). In the earlier tests the survival time of the altitude group was about two-thirds that of the control group, in agreement with the data of Table II. Thus degree of susceptibility to salmonellosis accompanying hypoxic stress appears to remain approximately constant with time of acclimatization ranging from 3 weeks to 4 months. The correlation in time of change of tissue citrate with change in susceptibility to infection, previously noted(2), is now extended to include the 3- to 4-month period. That such a correlation between tissue citrate and susceptibility to infection is *not* to be expected in all situations leading to greater susceptibility is seen in the results with cortisone. Cortisone injections greatly reduce survival time of infected mice(5) yet fail to alter in all cases tissue citrate. This action of cortisone on infections has been confirmed with our strain of *Sal. typhimurium* and CF 1 mice.

Summary. 1. Citric acid content (γ/g wet wt) of blood, diaphragm, duodenum, heart, kidney, liver, lung, and spleen was determined colorimetrically in the following groups of mice: (a) normal mice, (b) mice exposed to simulated altitude of 20,000 ft for 3 to 4 months, (c) mice given daily intraperitoneal injections of 1 mg cortisone acetate for 2 weeks, and (d) mice starved for 17 hours. 2. Mice of group (b) had 20-30% less tissue citrate than control mice. In group (c) there was no change in tissue citrate except in kidney, which increased, and in spleen, which decreased. Tissues from starved animals showed a significant increase in citrate in all tissues. Thus the change observed in group (b) cannot be correlated with cortisone injections nor with a reduced blood sugar level induced by inanition. 3. Altitude exposed mice are more susceptible to *Sal. typhimurium* infection than control mice.

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Relation of Number of Transplants to Total Volume of Regenerating Adrenocortical Tissue.* (23445)

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Wyman and tum Suden(1) found that the total amount of adrenocortical tissue regenerating in intramuscular autoplastic transplants in rats is fairly constant for each sex, irrespective of number of masses implanted or present, and suggested that a functional factor limits the growth of transplants. Eversole, Edelmann and Gaunt(2) pointed out the considerable variability in amount of tissue regenerating from single grafts in different sites and suggested that the principle stated by Wyman and tum Suden would hold only for a given site. In the Wyman and tum Suden study the transplants were measured during the third month after operation or later. Komrad and Wyman(3) found that resumption of function of adrenal transplants, beginning about one week after operation, proceeds in a progressive or stepwise manner, presumably reflecting an increase in volume of the secreting tissue. In a study of the effect of x-irradiation on growth of transplants(4) considerable variation in size of grafts was noticed at 14 days postoperation. An attempt was made, therefore, to assess the relation of number of implants or of surviving transplants to total volume of regenerating adrenocortical tissue in intramuscular grafts during the earlier stages of regeneration, and to find the time at which a limitation of growth appears.

Methods. Male rats of the Wistar strain, 35 days of age, weighing 80 to 120 g at operation, were maintained on Purina laboratory chow and tap water *ad libitum*. After operation saline drinking water was given for the duration of the experiment. The bisected adrenal glands were transplanted to the spinotrapezius muscle. Approximately equal numbers among the 56 rats used received one, 2, 3, or 4 implants of half a gland each. The rats were sacrificed at 9, 14, 21, or 90 days after operation and the number of regenerated transplants was determined. The fixed (Gilson's) tissues were serially sectioned in paraf-

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TABLE I. Relation of Number of Transplants to Total Volume of Regenerating Adrenocortical Tissue in Rats.

Age of transplant (days)	No. of transplants growing	No. of rats	Avg vol/rat (mm^3)	Avg vol/transplant (mm^3)	Avg cell depth/transplant	Avg cell depth/age group*
9	1	4	.05	.05	9.3	15.7
	2	4	.38	.19	15.3	
	3	3	1.1	.38	20.6	
	4	2	1.4	.34	14.1	
14	1	4	.27	.27	17.8	36.0
	2	5	1.6	.82	34.9	
	3	3	2.3	.76	31.1	
	4	4	3.0	.74	30.4	
21	1	4	2.5	2.5	55.5	58.8
	2	6	2.7	1.4	48.6	
	3	3	3.3	1.1	44.9	
	4	3	6.4	1.6	52.3	
90	1	3	7.0	7.0	74.0	89.1
	2	4	11.3	5.7	93.8	
	3	3	12.4	4.1	74.8	
	4	1	11.0	2.7	64.2	
Sham operated rats: intact adrenals	Day 9	3	15.0	7.5	72.6	82.0
	" 14	3	15.3	7.7	75.4	
	" 21	4	20.7	10.3	72.1	
	" 90	1	18.3	9.1	88.0	

* Calculated from greatest cell depth/animal.

fin at 24 μ and stained (H & E). Each section was projected, outlines of the adrenocortical masses were traced, the areas were measured (sq mm) by planimetry, and volumes (cu mm) were calculated. The volume of cortical tissue in the adrenals of 11 sham operated rats of the same postoperative ages was similarly determined. Cell depth measurements were made by counting the number of nuclei in a straight line between the capsule and the inner edge of the largest mass of regenerated tissue in the transplant.

Results. The volume of regenerated adrenocortical tissue increased with age but at 90 days after operation it was still less (about half) than that present in intact adrenal glands (Table I). This may have some relation to the intermediate position between intact and adrenalectomized animals that transplanted rats exhibit with respect to function as tested by stress tests(3). The cell depth at 90 days, however, was about the same as that of intact adrenals so the difference in volume resulted from the difference in form between the discrete single masses of the transplants and the hollow shell of an intact cortex. The equivalence of cell depths may indicate that at this time the transplants had reached the limit of their regenerative capacity.

During the first 2 weeks after operation, total volume of tissue per rat increased with the number of transplants regenerating while, except for the one transplant animals which lagged behind, average volumes and cell depths per transplant were roughly equivalent. At 21 days, however, the rats with one, 2, or 3 transplants had about the same total volume of tissue while the volumes per transplant showed an inverse relationship to number of grafts. The 4-transplant group had about twice as much tissue. The cell depths for all 4 groups were about the same. At 90 days the total volume of regenerated tissue per rat was about the same irrespective of number of transplants, so volumes per transplant for the entire series were inversely proportional to number of grafts. Again the cell depths for the 4 groups showed rough equivalence. In the sham operated control animals the volumes of their intact adrenals increased somewhat with increasing age but not remarkably, reaching a maximum at 21 days after operation when the rats were about 56 days old.

Discussion. From these data we may conclude that during the first 2 weeks after intramuscular implantation adrenocortical grafts while progressively resuming their function, are unable to deliver enough cortical secretion to establish the pituitary-adrenal equilibrium which is thought to regulate size and performance of adrenocortical tissue(1). After 2 weeks, however, the equilibrium becomes established, imperfectly at 21 days but completely at 90 days, and the amount of cortical tissue which can regenerate becomes more a function of time than of the number of regenerating masses.

Summary. The total volume of adrenocortical tissue regenerating in intramuscular autotransplants in rats during the first 2 weeks after operation is proportional to number of transplants growing. Thereafter, the total volume is about the same irrespective of the number of implants made or transplants regenerating, so the volumes per transplant are inversely proportional to the number of grafts. Average volume of regenerating tissue and cell depth increases with increasing age of transplant. At 90 days after operation the cell depth is like that of intact adrenal glands but the volume is less. Apparently transplants cannot deliver the amount of cortical secretion necessary to establish a pituitary-adrenal equilibrium until after 2 weeks of growth.

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Effect of Insulin on Clearance of Emulsified Fat from the Blood in Depancreatized Dogs.* (23446)

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The metabolic abnormality that underlies development of lipemia has not been determined. The frequent occurrence of this condition in association with uncontrolled diabetes and its disappearance with treatment suggests that carbohydrate metabolism is in some way linked to development of lipemia. Similarly, the presence of extreme lipemia in patients with von Gierke's disease seems indicative that abnormal carbohydrate metabolism is in some manner related to lipemia. Since diabetes and von Gierke's disease represent different disorders it is apparent that lipemia may arise from various metabolic disturbances. The blood lipids in these diseases are generally considered to be of exogenous origin(1) although this has not been proven and is in fact, contradicted by numerous clinical examples of severe and worsening lipemia despite a diminished food intake. In the diabetic there is rough parallelism between fasting blood sugar and degree of lipemia(2). Inasmuch as fat utilization is increased in the diabetic state, there has been no reason to suspect that failure to oxidize fat by liver and extrahepatic tissues could cause high-fat levels in the blood. Neither is there any connection between impairment of fat synthesis in diabetes(3) and the lipemic state. Stanley and Thannhauser(4) and Lever and Waddell (5) have indicated that there is slowed passage of fat from the blood in patients with idiopathic hyperlipemia.

It has long been known that insulin lowers the fat and cholesterol levels of the blood(1) and that it will prevent elevation of blood lipids and hepatic fat accumulation in ani-

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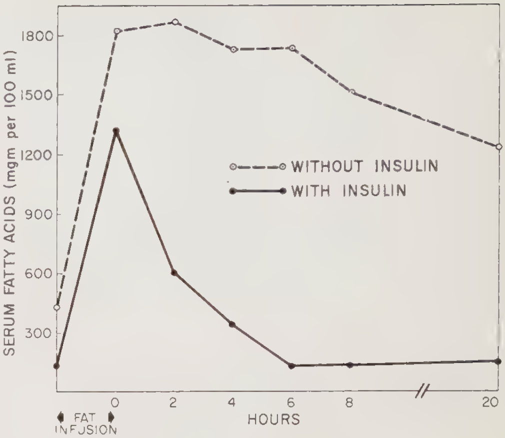
mals with phlorizin diabetes(8). Insulin administration to diabetic patients also flattens and shortens alimentary lipemia curves(2). In an attempt to clarify this problem the clearance of injected fat emulsion from the blood has been studied in normal dogs and in pancreatectomized dogs with and without insulin. It has been shown that pancreatectomized animals maintained on insulin can clear injected fat from the blood normally whereas deprivation of insulin results in delayed clearance of all lipid components and a rise in cholesterol. These experiments confirm and extend the observations of Lombrosa(6) and Rony and Mortimer(7).

Methods and Material. Male mongrel dogs weighing 10 to 20 kg were fed a constant diet consisting of 1 lb of horse meat, 1 qt of milk and 50 g of sugar plus supplements of iron and vit. B complex. After 2 weeks of this diet a fat tolerance curve was run. For this the animals were fasted for 24 hours, then infused with a 10% cottonseed oil emulsion which contained soyabean phosphatide 1.2% and Pluronic F68 0.2% by weight(9). This was administered intravenously at a rate of 5 ml per minute. Twenty ml per kg of body weight were given. Throughout the next 20 hours the animals were fasted. Venous blood samples were drawn before infusion, at the end of infusion and at 2, 4, 6, 8, and 20 hours following the completion of infusion. On each of these samples the following determinations were made—turbidity(10), total fatty acids(11), phospholipid(12), total and esterified cholesterol(13). Neutral fat concentrations were calculated by the method of Peters and Man(14). Total pancreatectomy was then carried out on 5 dogs. After a 2- to 3-week period of convalescence and regulation of diabetes another fat clearance study was performed exactly as before the operation. Each dog lost weight during this period so the amount of fat injected was reduced accordingly. The animals received their regular dose of NPH insulin (8-15 units) on the day of this test. One week later another fat clearance test was carried out in exactly the same manner except that insulin was omitted the day preceding and day of this test.



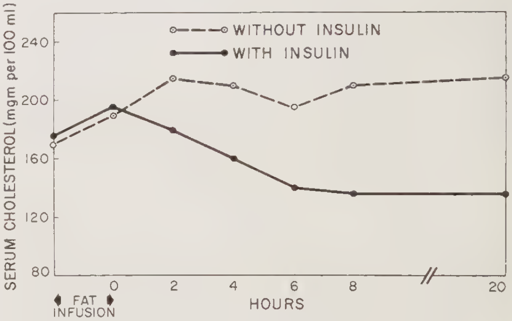


FIG. 1 (top). Serum fatty acid during and after fat infusion with and without insulin in a pancreatectomized dog.

FIG. 2 (bottom). Total serum cholesterol during and after fat infusion into a pancreatectomized dog.

Results. The passage of fat from the blood stream is impaired in the animals without insulin whereas in those receiving insulin the rate of removal of fat from the blood proceeds at a normal or elevated rate. (Tables I, II, III, Fig. 1, 2.) These results are not compatible with the concept that over-mobilization of endogenous fat is the primary cause of lipemia in uncontrolled diabetes. The amount of fat introduced into the blood was the same in each group of animals, therefore it seems clear that there was impairment in the removal of this fat from the blood.

Direct relationships between insulin and fat metabolism are not known, whereas a number of indirect ones mediated through the action of the hormone on carbohydrate metabolism are well established. It can be seen from the data in Fig. 1 and 2 and Table I

TABLE I. Normal. Turbidity and serum lipids in 5 normal dogs before and after emulsified fat infusions. Except for turbidity the numbers express conc. in mg/100 ml. Upper figure is the mean and lower figures give ranges.

	Pre	0 hr	2 hr	4 hr	6 hr	8 hr	20 hr
Turbidity	2.1	85.6	44.5	27.3	16.8	9.6	2.9
	1.0–3.7	54.8–146	32.0–60.7	14.3–43.0	8.2–24.7	2.7–14.4	1.4–5.6
Fatty acids	328	1473	1036	721	638	476	350
	223–337	1230–1800	973–1175	563–804	368–810	380–557	288–435
Neutral fat	3	1143	684	360	282	124	9
	0–8	895–1390	635–738	224–470	48–400	65–267	0–28
Phospholipid	361	401	395	373	363	357	361
	327–387	372–430	365–430	357–400	357–400	335–400	320–430
Total cholesterol	270	270	282	272	262	255	243
	235–330	220–320	245–355	235–330	220–320	210–320	190–320
Ester cholesterol	188	200	193	185	177	170	162
	150–245	165–235	165–245	160–225	150–220	140–220	115–235

TABLE II. Pancreatectomy with Insulin. Turbidity and serum lipids in 5 pancreatectomized dogs receiving insulin. Except for turbidity the numbers express conc. in mg/100 ml. Upper figure is the mean and lower figures give ranges.

	Pre	0 hr	2 hr	4 hr	6 hr	8 hr	20 hr
Turbidity	1.9	67.0	36.7	17.2	6.7	3.9	1.2
	1.7–2.3	49.0–98.0	19.8–66.8	4.5–46.8	2.5–14.6	1.5–7.1	1.0–1.5
Fatty acids	299	1428	845	505	319	277	256
	226–450	905–1725	610–1150	307–900	201–470	195–372	215–349
Neutral fat	68	1141	575	263	90	64	34
	9–122	62–1390	350–845	71–688	0–267	0–173	6–65
Phospholipid	271	359	322	283	260	248	250
	227–392	320–490	247–495	217–465	200–435	190–400	183–340
Total cholesterol	185	184	193	183	158	156	156
	150–245	140–230	165–230	160–230	140–265	115–210	120–200
Ester cholesterol	85	117	109	99	87	92	86
	40–140	90–160	80–140	75–140	65–130	60–150	60–135

TABLE III. Pancreatectomy without Insulin. Turbidity and serum lipids in 5 pancreatectomized dogs without insulin. Except for turbidity the numbers express conc. in mg/100 ml. Upper figure is the mean and lower figures give ranges.

	Pre	0 hr	2 hr	4 hr	6 hr	8 hr	20 hr
Turbidity	2.0	60.8	57.2	47.9	43.1	38.6	13.5
	0–5.6	50.6–70.1	43.9–77.7	35.3–62.0	31.7–57.0	25.7–53.0	3.4–25.4
Fatty acids	332	1569	1785	1525	1152	1279	1003
	255–428	1125–2010	1395–2470	1190–2020	995–1735	710–1840	460–1500
Neutral fat	110	1287	1503	1260	949	1022	740
	40–238	860–1730	1145–2160	945–1730	750–1495	465–1575	258–1185
Phospholipid	288	379	367	344	345	332	354
	240–320	333–410	305–400	302–365	295–367	288–350	243–440
Total cholesterol	188	180	216	230	212	212	197
	165–245	165–210	195–280	195–300	195–265	195–265	165–255
Ester cholesterol	72	104	117	105	98	97	77
	45–110	85–130	95–150	65–145	75–130	85–115	45–110

that all lipid components reflected the withholding of insulin from the depancreatized animals. Thus, there was no evidence to suggest that the primary defect was in any particular lipid fraction. One of the striking effects of diabetes on lipid metabolism is the almost complete inability to synthesize fatty acids. The same phenomenon is found in normal animals when fasted for 24 hours. If this block in fatty acid synthesis was the underlying reason for the delayed removal of fat from the blood, fasted normal animals should also display this retardation. Such was not the case, however. In contrast to retarded fatty acid synthesis is the accelerated formation of ketone bodies which occurs in diabetes(15). It is possible that excessive ketones may delay fat clearance because of an influence on cellular physiology in addition to those recognized and characterized chemically(16).

If this reasoning is correct then the problem becomes more clearly one of membrane permeability (in the liver) to hydrophobic lipid molecules, aggregates and particles. It seems possible that in the diabetic animal deprived of insulin at least 2 facets of abnormal carbohydrate metabolism might lead to impairment of the passage of negatively charged lipid particles into the liver. First, decreased energy production from Krebs cycle oxidations may alter membrane potential directly (17) and secondly the acidosis that is invariably present might have a similar effect. To test the latter possibility 3 pancreatectomized dogs receiving insulin were made acidotic by infusion of ammonium chloride solution prior to and during the fat infusion. This resulted in slowed fat clearance curves identical to the uncontrolled diabetic dogs. The possibility remained that ammonium ion toxicity itself was responsible since it has been shown *in vitro* that ammonium causes increased ketone body production due to interruption of the Krebs cycle and diversion of ketone body precursors in the direction of acetoacetate(18, 19). In normal dogs slow administration of ammonium chloride or more rapid administration of ammonium carbonate had no effect upon rate of fat clearance. It would thus appear that the effect of ammonium chloride is more likely due to acidosis. Furthermore in 2 pancreatectomized dogs administration of dilute hydrochloric acid caused slowing of fat clearance from the blood even though insulin was given.

In Table I and Fig. 1 and 2 it can be seen that infusion of fat emulsion into the group of pancreatectomized animals receiving insulin and also in normal animals resulted in a decrease in serum cholesterol concentrations which persisted through the 20-hour period of observation. The reverse was true in the pancreatectomized animals deprived of insulin. This cholesterol lowering effect has been observed in patients receiving similar fat emulsions intravenously(5). This phenomenon appears to be contingent upon normal carbohydrate utilization and it seems justifiable to suggest that in these experiments is the *in vivo* counterpart of the experiments of Hotta, Hill and Chaikoff(20,21), who observed that acetate-1-C^{14} was incorporated into cholesterol at an increased rate by the livers of diabetic rats as compared to normal, and that insulin treatment of diabetic animals resulted in diversion of acetate into metabolic paths other than cholesterol synthesis. It seems likely that the rise in cholesterol observed after infusion of fat into diabetic animals is a reflection of the same phenomenon.

Since no direct experimentation concerning the function of clearing factor in diabetic subjects has been reported there is insufficient evidence for evaluating the possible relationship to the above experimental results. It seems possible that enzyme synthesis or physiologic activity might be influenced by abnormal carbohydrate metabolism but from a quantitative point of view the likelihood of clearing factor activity being an important facet of the above described phenomena appears quite remote.

Summary. Emulsified fat infused into pancreatectomized dogs is cleared from the blood at a normal rate if the animal is receiving insulin whereas without insulin the injected fat is cleared slowly. It thus appears that the clearance of fat from the blood is dependent upon normal carbohydrate utilization. Serum cholesterol levels rise follow-

ing infusion of fat in diabetic animals deprived of insulin. Serum cholesterol concentration falls following infusion of fat into pancreatectomized animals receiving insulin. Observations on fat metabolism in which the fat is administered intravenously give data comparable to those obtained following oral administration of fat and the mechanisms of clearance seem identical by the two methods.

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Amperometric Determination of Sulfhydryl Changes in Mouse Epidermis and Dermis During Treatment with Certain Polycyclic Hydrocarbons.*

(23447)

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Investigation of sulphur metabolism in biological systems is challenging because of its integration with cell activities. Currently, the relationship between sulfhydryl groups and the carcinogenic process is receiving much attention. Polycyclic carcinogenic hydrocarbons have been presumed to interfere with some phase of sulphur metabolism related to growth. Reimann and Hall(1) showed that parathiocresol inhibited induction of tumors by 1,2,5,6-dibenzanthracene. They concluded that the sulfhydryl group of parathiocresol had an anti-carcinogenic effect. Fieser(2) discovered a close relationship between carcinogenicity and certain types of substitution reactions at positions in the molecule. He suggested a reaction of the hydrocarbon *in vivo,* whereby an S-S linkage of a protein or peptide is broken, giving rise to a free sulfhydryl group and a hydrocarbon-peptide link. Later, Crabtree(3,4) suggested that during carcinogenesis, inhibition may occur by some interference with sulphur metabolism. Crabtree postulated that in the production of a skin tumor by a carcinogen, the initial step involved was the fixation of the carcinogen by combination with free sulfhydryl groups.

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Others(5) have demonstrated that the carcinogenic hydrocarbons react with RSH groups. DiPaolo, Hill and Stanger(6) reported that some carcinogenic and non-carcinogenic polycyclic hydrocarbons reduced the amount of soluble SH in treated skin of mice.

The purpose of the present investigation was to determine amperometrically the changes in the total sulfhydryl content, in protein sulfhydryl content, and by subtraction, in the soluble sulfhydryl content of mouse epidermis and dermis which were treated with certain carcinogens or with anthracene.

Materials and methods. The polycyclic carcinogenic hydrocarbons employed in this work were 1,2,5,6-dibenzanthracene (DBA), 9,10-dimethyl-1,2-benzanthracene (DMBA) and anthracene. The concentrations of DBA and DMBA were 0.05% (weight-volume%) in acetone solution and the concentration of anthracene was 0.10%. The mixture of two carcinogens consisted of 0.05% DMBA and 0.10% DBA in the same solvent.

Female Bagg albino mice 3½ months old were obtained from our own colony. Immediately before application of acetone solutions, hair was removed from the dorsal skin with electric clippers and the mice were selected in which the dorsal hair appeared to be in the telogen (resting) phase of the hair cycle. To secure an adequate sample a pair of mice was used for each determination. Two-hundredths ml of solution was applied to the clipped skin of each test mouse with a calibrated pipette. (0.02 ml of a 0.05% solution equal 10 μg of solute.) At selected intervals, pairs of mice were killed by cervical dislocation and the treated skin was promptly removed. The subcutaneous fat and connective tissue were scraped off with a scalpel. To separate dermis from epidermis, the scraped whole skin was placed on a piece of clean polyethylene sheeting. After the skin was stretched, the epidermis was scraped off with a scalpel(7). The epidermis and the dermis from each pair were minced separately with scissors, placed in a small beaker, and weighed. The mince was transferred to a Potter homogenizer immersed in ice water. The weight of the sample was determined by difference.

After being homogenized with 10 ml of glass-distilled water, the homogenate was transferred to a plastic centrifuge tube, and the washings from an additional 5 ml of water were added. The suspension was centrifuged in a refrigerated International Centrifuge, Model PR-2 with a high speed-head for 15 minutes at 18,000 rpms, at a temperature of 5°C. The supernatant solution was filtered with No. 41 Whatman filter paper. The total sulfhydryl content and the protein sulfhydryl content were determined by titration with 0.001 M $HgCl_2$ solution, according to the technic of Kolthoff, Stricks, and Morren(8). The primary reaction in the mercurimetric amperometric titration is the binding of one mercury atom by one SH group.

This procedure utilized a rotating platinum electrode (connected with a Sargent Ampot) inside a 50 ml beaker containing 15 ml of phosphate buffer(8). Pure nitrogen was bubbled through this solution until the residual current (with an applied EMF of $-0.2V$ *vs.* saturated calomel electrode) was less than 0.5 μamp. For total SH determination, a 5 ml aliquot of the water filtrate was added to 15 ml of phosphate buffer low in oxygen in a 50 ml beaker and titrated as above.

For the protein SH determination, 1 ml of 10 M trichloroacetic acid was added to a second 5 ml aliquot of water filtrate. This sample was centrifuged as before, the supernate discarded and the remaining protein precipitate dissolved by adding 1 ml of 1 N NaOH. Following neutralization of the sample with 2 N HCl, the solution was titrated amperometrically using phosphate buffer as previously described. The results were calculated as mg of SH per 100 mg net weight of either dermis or epidermis (one ml of 10^{-3} M $HgCl_2$ equals 0.033 mg SH). The accuracy of this technic was tested by titrating a standard solution of glutathione at frequent intervals.

Results. A summary of the mouse epidermis response to different polycyclic carcinogens topically applied is presented in Fig. 1. The changes observed in the dermis are in Fig. 2. The control values are indicated by the solid horizontal lines. (The figures represent the averages of the determinations at each time interval.)

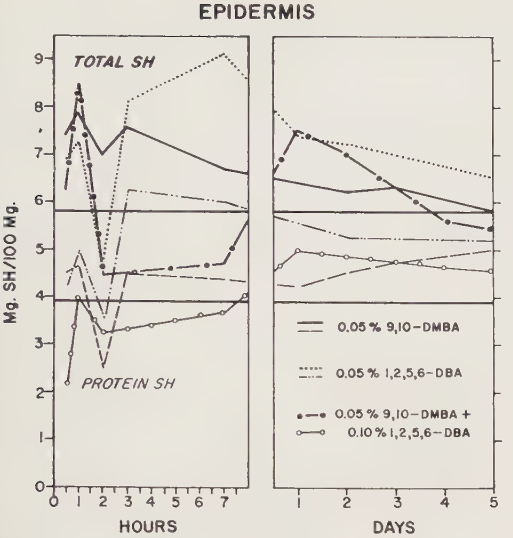

FIG. 1. Changes produced per 100 mg of mouse epidermis by one painting of a carcinogen or by a mixture of 2 carcinogens. Upper horizontal line is mean control value obtained for total sulfhydryl and lower horizontal line is mean control obtained for protein sulfhydryl.

At frequent intervals untreated mice whose dorsal hair had been clipped were used as controls for the epidermis and the dermis. The total sulfhydryl level of the epidermis and the dermis was 5.8 ± .57 mg/100 mg and 3.7 ± .47 mg/100 mg respectively; the protein sulfhydryl level of epidermis was 3.9 ± .57 mg/100 mg and that of the dermis was 2.4 ± .32 mg/100 mg. In both the epidermis and the dermis the protein sulfhydryl constitutes about 2/3 of the total sulfhydryl although it should be noted that concentration of total sulfhydryl in the dermis was about 2/3 as much as in the epidermis. By subtraction, the remaining 1/3 was considered soluble sulfhydryl. Painting the clipped skin of mice with the acetone solvent did not produce any change in the sulfhydryl levels measured.

Three compounds in acetone solution were tested for their effects on skin sulfhydryl levels. Two of these were the carcinogens DMBA and DBA. They were also tested in combination because DBA is known to inhibit skin carcinogenesis caused by DMBA(9). The last compound, anthracene, did not appear in either figure because the reactions to it were always within the untreated ranges. It was used as another control because it is a polycyclic hydrocarbon with no carcinogenic action on mouse skin(10).

The preliminary work on the soluble sulfhydryl level of whole skin demonstrated that a pronounced change occurred soon after painting. Consequently, in these experiments determinations were made at ½, 1, 2, 3, 7, and 24 hours and daily through 5 days.

Anthracene at double the concentration of the carcinogens used had no effect on either the sulfhydryl level of the epidermis or the dermis. The total sulfhydryl and the protein sulfhydryl levels were within the normal range at all intervals tested.

Epidermal changes. During the first hour total sulfhydryl content was above normal for all compounds tested (Fig. 1). The 0.05% DBA and the combination 0.5% DMBA-0.10% DBA caused a fall in total sulfhydryl content which reached its lowest point 2 hours after treatment. The low point in either case was about the same and averaged 4.5 mg/100 mg of epidermis or about 75% of the mean control value. At the third hour the level had begun to rise and individual differences became apparent. For instance, with DMBA, the total sulfhydryl level slowly returned to normal by the fifth day; the DBA reached a maximum of 9 mg of total sulfhydryl/100 mg of epidermis at 7 hours, slowly diminished and had not returned to normal at the fifth day. The most outstanding feature of the combina-

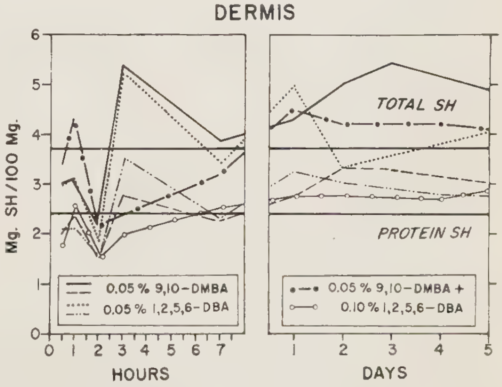

FIG. 2. Changes produced per 100 mg of mouse dermis by one painting of a carcinogen or by a mixture of 2 carcinogens. Upper horizontal line is mean control value obtained for total sulfhydryl and lower horizontal line is mean control obtained for protein sulfhydryl.

tion was the sustained decline for several hours. The effect on the protein sulfhydryl for the most part paralleled the corresponding total sulfhydryl curve with the following effect at the 2 hour levels: with DMBA, 2/3 of normal; with DBA barely below the mean control; with the combination, 15% below the mean control. In all 3 situations the protein sulfhydryl level was still above the mean control value at 5 days.

Dermal changes. In Fig. 2 are presented the changes which occurred in the dermis. At the first hour the total sulfhydryl was above normal with the combination of DMBA-DBA only; however, all 3 curves were depressed by the second hour. The individual carcinogens attained a maximum at 3 hours. The weaker carcinogen, DBA, had a second peak at 1 day while the stronger carcinogen had a second peak at 3 days. The combination of both carcinogens had only one peak. Its maximum was not as high as either carcinogen alone. The protein sulfhydryl results were lower but similar to those obtained for total sulfhydryl.

Soluble Sulfhydryl. The soluble sulfhydryl obtained from the total sulfhydryl and protein sulfhydryl reflected the same changes described above. For example, at the 2 hour level soluble sulfhydryl levels were down ⅓ for the mixture of 2 carcinogens and for the DBA while the strong carcinogen was 250% of normal. The drop in soluble sulfhydryl was greater in the dermis and was equal to ⅓ of the control in the case of DBA and ½ of the control in the other 2 series. The falls in total sulfhydryl and protein sulfhydryl in the dermis are so similar as to indicate that very little change occurs in the soluble sulfhydryl of the dermis. It must be concluded that most of the soluble sulfhydryl change takes place in the epidermis.

Discussion. The activity of the carcinogenic agents on the sulfhydryl level observed in these experiments with mouse skin is in agreement with those reported by Wood and Kraynek(11) on effect of 3,4 benzpyrene on the plasma sulfhydryl in the dog. The sustained drop in sulfhydryl produced by the combination of the 2 carcinogens cannot be attributed to the total concentration of hydrocarbons. When either carcinogen was used at a concentration of 0.15%, the response pattern was similar to that obtained with lower concentration. No explanation is offered for the difference in response obtained by DMBA and DBA. Using an indirect method of determining glutathione, Crabtree(12) was not able to demonstrate an effect on the soluble sulfhydryl level of skin by 3,4 benzpyrene and DBA. Nor was there any indication of utilization in the end product of metabolism. Crabtree did report that naphthalene, phenathrene, and to a slight extent anthracene, when given in large doses decreased tumor induction by 3,4 benzpyrene or DBA and caused a decrease in the SH content of skin followed by a prompt return to the normal level.

Many approaches have been used to elaborate the place of -SH groups in carcinogenesis and growth. As early as 1931 Reimann(13) noted that SH groups caused increased differentiation and organization of cells. He postulated that a qualitative change in chromosomal chemical groups which controls differentiation and organization must occur before malignancy. According to Davidson and Lawrie(14) chromosomes are poor in sulfhydryl groups and the oxidation of such groups might be responsible for somatic mutations concerned in the initiation of cancer(15).

A comprehensive study on the role of SH groups in both chemical carcinogenesis and anticarcinogenesis was made by Crabtree(3). He showed that a number of compounds which are known to react with -SH group containing cell constituents can act as anti-carcinogens. He has suggested that carcinogens become attached to cellular constituents of the skin of mice only in the presence of -SH groups and that many inhibitors of carcinogenesis cause local depletion of sulphur in the skin.

The observations reported here do not offer an adequate explanation of the mechanism involved. The possibility has been ruled out that the maximal dilution reported might be actually a dilution factor due to edema. The wetbacks of control animals and animals treated with carcinogen or inhibitor did not show any significant weight differences when sacrificed at two hours and dried to constant weight at 105°C. There are, however, a number of problems to be considered. Perhaps the only effect on these agents is to speed the metabolites of the sulfhydryl group out of the tissue or to prevent the production of these sulfhydryl groups. This assumes a rather high rate of metabolism of sulfhydryl by the skin. The possibility that there is a binding of these agents with sulfhydryl groups must also be considered.

Summary. An amperometric titration method for determining sulfhydryl groups was applied to mouse skin. The total sulfhydryl, the protein sulfhydryl and by subtraction, the soluble sulfhydryl contents were determined after painting with anthracene, DBA, DMBA, or a combination of the 2 carcinogens. The anthracene and the solvent, acetone, had no effect on the sulfhydryl levels. In general the shape of the curve of the total sulfhydryl content of the epidermis and dermis was the same, although the dermis contained approximately half that of the epidermis. For the most part, the effect on the protein sulfhydryl paralleled the corresponding total sulfhydryl curve. Most of the change in the soluble sulfhydryl level is presumed to occur in the epidermis. No cause and effect relationship to carcinogenesis or anticarcinogenesis is supported by these observations on the sulfhydryl groups of the skin.

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Antifibromatogenic and Antihysterotrophic Activities of Synthetic Androgens (19-nor-methyltestosterone, 19-nor-testosterone phenlypropionate, Δ^1-testosterone and Δ^1-androstenedione).* (23448)

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Both the progestational and antiestrogenic (antifibromatogenic, antihysterotrophic, antiluteinizing) activities of 19-nor-progesterone are greatly superior to those of progesterone (1,2,3). The same is true for 19-nor-ethinyltestosterone(4,5,6,7). The progestational activity of 19-nor-methyltestosterone in women is considerable(8); it is active by oral administration(9). Various activities of 19-nor-testosterone and of its esters also are remarkable (10). Results with the antifibromatogenic and antihysterotrophic activities of 19-nor-methyltestosterone (nMT) and of 19-nortestosterone phenylpropionate (nTpp) are given in the present paper. Results obtained with Δ^1 derivatives of androstene also have been included. The glucocorticoid activity of Δ^1-hydrocortisone is several times that of

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* Cordial thanks are due to Dr. G. A. Overbeek, Department of Pharmacological Research, N. V. Organon, Oss, Holland, for a generous supply of the 4 synthetic androgens.

TABLE I. Antifibromatogenic and Antihysterotrophic Activities of 19-nor-Testosterone Phenylpropionate (nTpp) and 19-nor-Methyltestosterone (nMT) Compared to Those of Testosterone Propionate (Tp) and Methyltestosterone (MT); of Δ^1-Testosterone (Δ^1-T) and Δ^1-Androstenedione (Δ^1-A) Compared to Those of Testosterone (T) and Androstenedione (A); in 149 Castrated Female Guinea Pigs with Subcutaneously Implanted Pellets of Estradiol (Absorption: 46 to 65 μg/day). Duration of experiments: 3 mo. For classification of results see(12,13).

Steroid tested							
Compound	μg/day	No. of animals	Fibrous tumoral effect†		$Q_{2\text{-}3}$‡	Uterine wt	No. of pellets; conc. (%)
Tp*	215 (162–267)	18	1.8 ± .46		.6	2.7 (3.1– 4.2)	1; 100
nTpp	8 (7– 11)	5	6.1 ± 1.40	1.4§	2.5	3.9 (3.1– 4.7)	1; 20
nTpp	19 (14– 26)	15	2.7 ± .66		.5	3.6 (1.6– 5.5)	1; 40 & 2; 20
nTpp	38 (32– 51)	8	1.1 ± .20 ↓		0	2.9 (1.8– 5.1)	3; 20
MT	11 (2– 28)	13	6.2 ± 1.03		1.8	5.4 (2.2– 9.9)	1; 20 & 40
MT	255 (231–276)	7	.7 ± .18	4.0	0	4.1 (3.0– 6.4)	1; 80
nMT	15 (2– 28)	20	1.7 ± .40 ↓		.1	3.1 (1.0–12.2)	1-3; 20
T	51 (47– 53)	5	3.8 ± 1.52		1.0	3.6 (2.5– 4.7)	2; 40
T	300 (118–484)	14	2.8 ± .51		.6	3.2 (1.7– 4.9)	1-2; 80
Δ^1-T	65 (40– 93)	10	3.7 ± .72	2.7	.9	2.2 (1.6– 3.3)	1-2; 40
Δ^1-T	375 (244–534)	16	5.1 ± .72 ↓		1.6	3.6 (2.0– 6.7)	1-2; 80
A	422 (273–572)	8	3.3 ± .63	2.3	.7	3.5 (2.4– 5.1)	1-2; 80
Δ^1-A	396 (266–536)	10	6.3 ± 1.14 ↓		2.0	4.5 (2.5–10.7)	1-2; 80

* See(12), p. 152, Table 14. With corrections.

† Arbitrary values for No. and size of tumors, ranging from 0 to 12; see(12).

‡ Arbitrary values for large tumors only; see(13).

§ Significant difference.

hydrocortisone(11). Thus clinical interest is attached to the question of the antifibromatogenic activity both of Δ^1-testosterone (Δ^1-T) and Δ^1-androstenedione (Δ^1-A) and of the mentioned 19-nor-compounds.

Methods were the same as in our former paper(6).

Results obtained with nTpp and nMT are given in Table I to be compared with those obtained with testosterone propionate (Tp) and methyltestosterone (MT). Fig. 1 and 2 give the individual results obtained in each comparative group. With an average of 19 μg/day of nTpp the antifibromatogenic action approaches that of 215 μg/day of Tp; with 38 μg/day of nTpp a more definite result is obtained than with the 6-fold of TP (Fig. 1). Thus the antifibromatogenic activity of nTpp is about 6 times that of Tp.

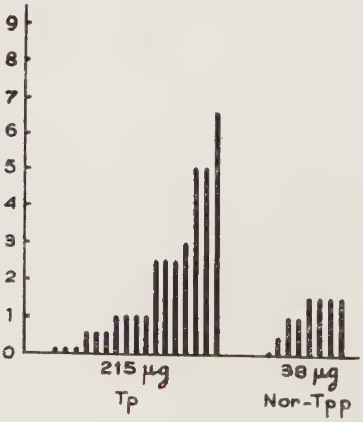

FIG. 1. Height of columns—F.T.E. of individual animals. Antifibromatogenic activity of 38 μg/day of 19-nor-testosterone phenylpropionate is equal and probably even superior to that of 215 μg/day of testosterone propionate.

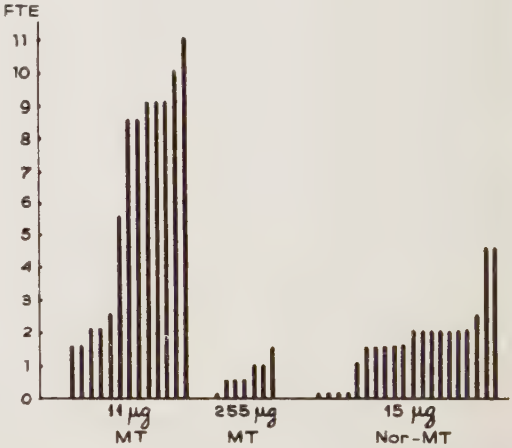

FIG. 2. Height of columns—F.T.E. of individual animals. With 15 μg/day of 19-nor-methyltestosterone almost the same result is obtained as with 255 μg/day of methyltestosterone; difference between 15 μg/day of the nor-cpd. and 11 μg/day of methyltestosterone is very striking.

No less clearcut were the comparative results with MT and nMT. The antifibromatogenic activity of 15 μg/day of nMT approaches that of 255 μg/day of MT (Fig. 2). With 11 μg/day of MT no antifibromatogenic action was obtained; 15 μg/day of nMT were sufficient to produce a very pronounced action, with a significant difference of 4.0 compared to 11 μg/day of MT. The antihysterotrophic activity of nMT, compared to that of MT, is even more remarkable; the small quantities of nMT used were more effective than the large quantity of MT. The antifibromatogenic activity of nMT is about 10 times that of MT.

Table I summarizes also the comparative results with the two Δ^1-cpds. The antifibromatogenic activity of the 2 cpds. decreases considerably in comparison to T and A (significant difference between Δ^1-T and T: 2.7). A decrease of the antihysterotrophic activity also is very probable.

Summary. The antifibromatogenic and antihysterotrophic activities of 19-nor-testosterone phenylpropionate and 19-nor-methyltestosterone are 6 to 10 times greater than those of testosterone propionate and methyltestosterone respectively. The activity of testosterone is decreased by Δ^1. No activity is obtained even with very considerable quantities of Δ^1-testosterone or Δ^1-androstenedione.

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Determination and Physiologic Disposition of Meprobamate.* (23449)

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Although the use of the tranquilizing agent 2-methyl-2-*n*-propyl-1,3-propanediol dicarbamate (meprobamate) has become widespread, relatively little is known about its physiologic disposition and metabolic fate. This report includes a specific method for determination of meprobamate in urine and the results of metabolic studies *in vivo* and *in vitro*.

Methods. Estimation of Meprobamate. Meprobamate is isolated from biological material by extraction into ether at an alkaline pH. After evaporation of the ether the amount of drug is determined spectrophotometrically at 450 mμ, at which wavelength the drug exhibits a pronounced peak when heated with sulfuric acid (Fig. 1). *Procedure.* To 3 ml of urine in a 15 ml glass-stoppered centrifuge tube are added 0.2 ml 1 N sodium hydroxide and 5 ml of ether.† Shake 5 minutes and centrifuge the tube. Two ml of the ether phase are evaporated to dryness under a stream of air in a heavy-walled test tube, and

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* A preliminary report was presented at Am. Chem. Soc., Miami, April, 1957.

† Ether was Mallinckrodt anhydrous analytical reagent.

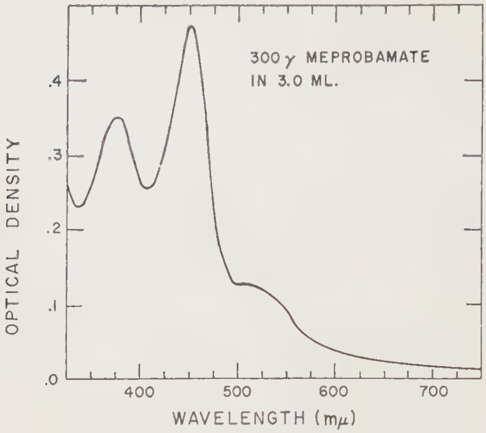

FIG. 1. Absorption spectrum of meprobamate after heating with sulfuric acid.

TABLE I. Molar Extinction Coefficient of Meprobamate and Related Compounds.

Compound	E_M*
2-Methyl-2-*n*-propyl-1,3-propanediol dicarbamate	9.2×10^4
2-Methyl-2-*n*-propyl-1,3-propanediol	1.1×10^4†
Methyl-*n*-propyl malonic acid	0
2-Methyl-2-*n*-propyl-3-hydroxypropyl carbamate	5.0×10^4
α-Methyl-α-*n*-propyl hydracrylic acid	0
α-Methyl-α-*n*-propyl-β-carbamoxy propionic acid	0

* Molar extinction coefficient based on difference between optical densities at 450 and 500 mμ for 1 cm path length.

† This material was not of high purity.

1 ml of concentrated sulfuric acid is added. The tube is heated at 100°C 30 minutes. After the solution is cooled, 2 ml of water are cautiously added to intensify the color. The mixture is allowed to stand for 15 minutes and the optical densities are measured in a Beckman DU spectrophotometer at 450 and 500 mμ. Since the ether extraction results in the recovery of only 85 ± 5% of the meprobamate, a standard solution containing 60 μg of meprobamate/ml is run at the same time as the unknown. The difference between the optical densities at 450 mμ and 500 mμ is used for meprobamate estimation in biological materials rather than the 450 mμ absorption alone in order to eliminate the interference of non-specific absorption by naturally occurring substances. The linearity of the color reaction is shown in Fig. 2. The sensitivity of the method can be increased by using smaller amounts of sulfuric acid and water and reading the optical density in a 1 ml cuvette. As little as 2 μg of meprobamate can be detected by this procedure.

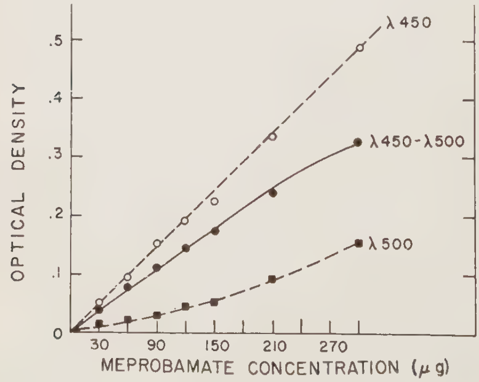

FIG. 2. Linearity of color developed in sulfuric acid reaction.

Results. Few compounds related to meprobamate reacted‡ with sulfuric acid (Table I). However, alcohols having 4 or more carbon atoms and no carboxyl groups showed the 450 mμ maximum when heated with sulfuric acid. For example, n-butyl and sec-butyl alcohols gave the reaction, but α, β, or γ hydroxybutyric acid did not.

Identification of meprobamate in urine. A test for meprobamate in the ether extract of urine of subjects who took the drug was positive. To establish the identity of the urinary product as meprobamate, an aliquot of the dried ether extract was subjected to the technic of comparative distribution ratios between a number of organic solvents and an equal volume of 0.1 N sodium hydroxide. It can be seen (Table II) that the apparent meprobamate isolated from urine and measured spectrophotometrically after sulfuric acid treatment had the same distribution ratios as the authentic compound, and was therefore presumably the same.

Urinary excretion. Four human subjects were each given a single dose of 1600 mg of meprobamate orally. Urine samples were collected from 0 to 24 hours and 24 to 48

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‡ The authors are indebted to Dr. B. Ludwig, Wallace Laboratories for samples of meprobamate and analogues.

TABLE II. Distribution Ratios of Apparent and Authentic Meprobamate.

Solvent	Authentic meprobamate	Apparent meprobamate isolated from urine
Ether	.94	.88
Ethylene dichloride	.82	.79
Benzene	.56	.58
n-Heptane	.31	.34

TABLE III. Urinary Excretion of Meprobamate in 4 Adult Human Subjects.*

Meprobamate excreted, mg		Total meprobamate excreted, % of dose
0-24 hr	24-48 hr	
159	33	12.0
148	43	11.9
234	96	20.6
145	49	12.1

* Each subject received 1600 mg of meprobamate orally.

hours and examined for free meprobamate (Table III). It can be seen that meprobamate is excreted relatively slowly for at least 2 days following a single dose. About 12 to 20% of the administered meprobamate was excreted unchanged in the urine. The cumulative urinary excretion in one of the subjects is shown in Fig. 3.

Metabolites of meprobamate. Berger(1) reported an increase in urinary glucuronides following ingestion of meprobamate. In order to obtain more direct evidence of glucuronide formation, urine from a subject given meprobamate was extracted with ether to remove free meprobamate and then incubated at 37°C with β-glucuronidase.§ Under these conditions, an ether-soluble substance was released that gave the characteristic meprobamate reaction with sulfuric acid. The urine incubated without glucuronidase or with boiled β-glucuronidase did not liberate any meprobamate-like material. The nature of the glucuronide formed has not been established, but the formation of an N-glucuronide appears unlikely, since this class of substances is more unstable than the urinary product obtained and is not hydrolyzed by β-glucuronidase(2). It is possible that β-hydroxylation occurs and that an O-glucuronide is formed. There is no evidence as yet that the carbamate linkage is altered metabolically. After treatment with β-glucuronidase, the meprobamate-like material was removed from the urine by ether extraction. Heating the residual urine with hydrochloric acid or sodium hydroxide resulted in the liberation of additional ether-soluble material that gave the characteristic meprobamate spectrum with sulfuric acid. The total amount of meprobamate-like material recovered, including substances released by the action of the β-glucuronidase and acid hydrolysis, account for about one-half of the administered dose, assuming that these products, if not identical with meprobamate, possess extinction coefficients in the color reaction of the same order of magnitude as meprobamate.

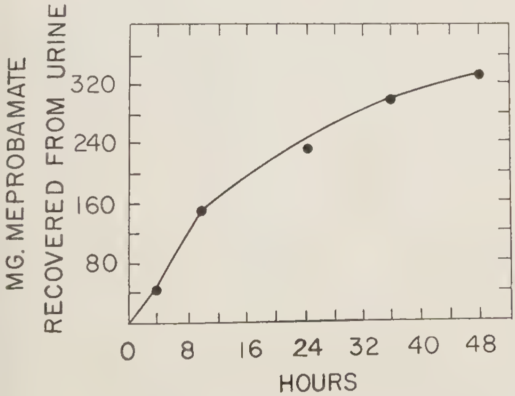

FIG. 3. Cumulative urinary excretion of meprobamate following oral administration of 1600 mg.

Metabolic studies with radioactive meprobamate. Meprobamate was tritiated by the technic of Wilzbach(3). The resultant product was diluted with nonisotopic meprobamate and recrystallized to constant radioactivity. The site of metabolic alteration of meprobamate was studied by incubating radioactive meprobamate with rat tissue slices as follows: 250 mg of tissue slice were incubated in an atmosphere of 95% O_2 - 5% CO_2

TABLE IV. Disappearance of H^3-Meprobamate after Incubation with Tissue Slices.

Tissue	Meprobamate disappeared, μM/g/hr
Brain	.205
Kidney	.302
Liver	.906
Hemidiaphragm	.330

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§ Ketodase, Warner-Chilcott Laboratories, N. Y.

in 2.5 ml Krebs-Ringer bicarbonate buffer containing 10 μM of glucose and 1.38 μM of radioactive meprobamate. At the end of 2 hours, the incubation mixture was homogenized, made alkaline, and the meprobamate was extracted with ether and counted in a liquid scintillation counter. The results shown in Table IV indicate that liver metabolizes the drug to a greater extent than do the other tissues examined. Following intravenous injection of 4 mg/kg of H^3 meprobamate significant levels of radioactivity were found in all tissue examined (liver, kidney, spleen, brain, testes, muscle, fat, spinal fluid, and blood).

Summary. (1) A specific method for the determination of meprobamate in urine is described. (2) Metabolic studies indicate that about 12 to 20 percent of the unchanged drug is slowly excreted in the urine in 48 hours and that additional products are formed, including several conjugates at least one of which is a glucuronide. (3) Radioactive meprobamate has been prepared and metabolic studies have been performed. The liver is the principal site of metabolic alteration.

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Effect of Pyrazinamide and Pyrazinoic Acid on Urate Clearance and Other Discrete Renal Functions.[*][†] (23450)

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Cullen *et al.*(1) and Gleason *et al.*(2) recently reported the occurrence of marked hyperuricemia, associated with decreased urinary urate excretion, after administration of the antituberculous agent pyrazinamide (pyrazinoic acid amide). The present report summarizes the results of clearance studies intended to determine more precisely the effect of the drug on the renal mechanisms regulating urate excretion. When it became apparent in the course of the study that the free acid (pyrazinoic acid) might be the active agent, appropriate studies with this compound also were initiated.

Methods. Fifteen male adults were available for study. Ten of these were gouty, in the intercritical phase of the disorder, and afforded opportunity to determine whether the drug could exert its effects in the face of already established hyperuricemia and, in some instances, excessive urinary urate excretion. To ascertain whether the renal effects occurred soon enough after oral administration of the drug to permit application of conventional renal clearance technics, preliminary experiments were performed in 5 subjects who received single oral doses of 3 g or 1 g. Determinations of the urate excreted in urine specimens spontaneously voided at hourly or 2-hourly intervals indicated a sufficiently prompt response, even with 1.0 g dosage. Standard renal clearance technics (3) were then employed in 10 subjects. After appropriate control periods, in which clearances of inulin, p-aminohippurate and urate were measured, the drug in 1 g or 3 g dosage was given by mouth; pyrazinamide in 4 experiments, pyrazinoic acid in 6 including 2 with 50 and 100 mg dosage. Post-medication urine collections, in 15-20 minute periods,

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[†] We are indebted to Dr. C. E. Lyght, Merck Sharp & Dohme Research Laboratories, for generous supply of pyrazinamide and pyrazinoic acid.

TABLE I. Time of Onset and Duration of Effect of Orally Administered Pyrazinamide on Serum and Urinary Urate in 2 Gouty Subjects.

		Urate	
Subject	Time (hr)	Serum (mg %)	Urine (mg/hr)
H.G.	–2	8.0	13.6
	Pyrazinamide, 3 g oral dose		
	2	10.5	11.4
	4	9.8	3.0
	6	9.8	1.3
	10	—	1.5
	18	—	2.4
	24	10.2	—
F.C.	–2	9.6	19.1
	Pyrazinamide, 1 g oral dose		
	1	—	20.7
	2	—	9.1
	3	—	2.6
	4	—	3.7
	6	—	7.8
	12	—	9.0
	24	12.8	—

were continued for 2-3 hours. In 3 experiments the effect of the drug on Tm_{PAH} was measured. Inulin, PAH and urate were determined by the standard methods indicated elsewhere(4); many duplicate urate determinations were made also by ultraviolet spectrophotometry. Ultrafiltration of serum urate before and after medication was performed as described elsewhere(5). Pyrazinoic acid amide and pyrazinoic acid concentrations in urine were measured by the method of Allen *et al.*(6).

Results. 1. *Time of onset and duration of response to orally administered pyrazinamide.* Table I summarizes the results of 2 representative experiments in gouty subjects given single doses of 3 and 1 g pyrazinamide respectively by mouth. Reduction in urinary urate excretion was apparent within 2 hours, reached a maximum in 2-6 hours, and persisted for the duration of the observations, 12-18 hours. The serum urate level rose within 2 hours and was still elevated after 24 hours.

2. *Effect of pyrazinamide on C_{urate}, C_{inulin}, C_{PAH}, Tm_{PAH}.* In 3 experiments with 1 g dosage of pyrazinamide, UV_{urate} consistently showed some decrease in 15-40 minutes and progressively declined over the 2 hours of observation. There was no significant increase in plasma urate in this period. C_{urate} fell correspondingly, from a pre-medication mean of 6.2 ml/min to a mean minimum of 1.8 ml/min, a decline to 29% of the initial value during the period of observation. There was no decrease in C_{inulin}, consequently the ratios C_{urate}/C_{inulin} paralleled C_{urate}. C_{PAH} fell from a mean of 485 to 390 ml/min. Tm_{PAH} was not affected. Table II cites the results in representative experiments. In 3 g dosage the effects were similar but more pronounced (Table II). C_{urate} fell from 8.9 to 0.5 ml/min, or 5.5% of the initial level. There was a decline in C_{inulin} and C_{PAH}.

3. *Effect of pyrazinoic acid on C_{urate}, C_{inulin}, C_{PAH}, Tm_{PAH}.* In 3 experiments with 1 g dosage of pyrazinoic acid, UV_{urate} consistently showed a sharp decline in 15-30 minutes and reached minimum levels in 15-60 minutes (Table II), without any change in plasma urate during this period. C_{urate} fell precipitously from a pre-medication mean of 10.1 ml/min to a mean minimum of 1 ml/min. There was no change in C_{inulin} or C_{PAH}. Tm_{PAH} fell somewhat, from 73.7 to 55.5 mg/min in Case J.F. and from 65.1 to 53.1 mg/min in Case V.E. In 3 g dosage the effects of pyrazinoic acid were essentially the same (Table II). Reduction in C_{urate} could be effected by pyrazinoic acid in doses considerably less than 1 g. Thus in one experiment a single orally administered dose of 100 mg lowered C_{urate} from 14.9 to 6 ml/min (40% of the initial value) and a second dose of 100 mg given one hour later reduced C_{urate} to 3.3 ml/min (22% of the initial value). An experiment in which 50 mg was given orally resulted in a fall in C_{urate} from 8.1 to 4.7 ml/min (58% of the initial value).

4. *Rate of urinary excretion of pyrazinoic acid and pyrazinamide.* It is apparent from the preceding data that pyrazinamide and pyrazinoic acid are rapidly absorbed from the gastrointestinal tract. In the body, pyrazinamide is largely hydrolyzed, pyrazinoic acid being the principal primary product of hydrolysis(6). Urinary excretion of the drug is slow. After oral administration of 1 or 3 g of pyrazinamide, less than 1% of the administered dose appeared unchanged in the urine in 2 hours, 2-3% as the free acid. Pyrazinoic acid is more rapidly excreted by the kidneys, 25-30% appearing in the urine in 2 hours

TABLE II. Results of Representative Experiments Employing Clearance Technics (Values Corrected to Standard Surface Area, 1.73 M^2).

Subject	Period (min.)	UV_{urate} (γ/min.)	C_{urate} (ml/min.)	C_{inulin} (ml/min.)	C_{urate}/C_{In} (%)	C_{PAH} (ml/min.)	Tm_{PAH} (mg/min.)
L.W.*	–45 to 0	470	4.5	86.4	5.2	332	
	Pyrazinamide, 1 g given orally						
	0-15	464	4.4	87.5	5.0	351	
	15-30	325	3.1	81.5	3.8	308	
	30-75	150	1.4	87.9	1.6	329	
	75-120	138	1.3	87.5	1.5	292	
M.E.*	–135 to –90	575	5.3	103	5.2	567	
	– 30 to 0	599	5.5	94.0	5.9		67.1
	Pyrazinamide, 1 g given orally						
	0-40	525	4.8	94.4	5.1		68.1
	40-80	366	3.4	90.7	3.8		63.8
	80-100	327	3.0	95.0	3.2		74.5
	100-120	313	2.8	100	2.8		72.8
S.Z.*	–45 to 0	994	8.9	125	7.1	505	
	Pyrazinamide, 3 g given orally						
	0-32	623	5.5	90.6	6.1	392	
	32-60	210	1.9	92.5	2.1	410	
	60-90	125	1.1	92.5	1.2	365	
	90-120	64	.6	84.0	.7	408	
	120-180	55	.5	80.0	.6	386	
D.C.	–45 to 0	486	7.3	111	6.6	544	
	Pyrazinoic acid, 1 g given orally						
	0-15	415	6.2	106	5.9	550	
	15-30	55	.9	101	.9	515	
	30-60	60	.9	105	.9	520	
	60-89	65	1.0	100	1.0	520	
	89-120	66	1.0	100	1.0	528	
J.F.*	–135 to –90	733	7.9	124	6.4	479	
	– 30 to 0	914	9.8	120	8.1		73.7
	Pyrazinoic acid, 1 g given orally						
	0-19	827	8.9	122	7.3		73.7
	19-38	195	2.1	140	1.5		70.5
	38-58	126	1.3	132	1.0		59.8
	58-98	119	1.3	139	.9		58.7
	98-158	128	1.3	123	1.0		55.5
J.A.	–45 to 0	554	6.8	118	5.8	443	
	Pyrazinoic acid, 3 g given orally						
	0-15	540	6.4	115	5.6	439	
	15-30	92	1.1	121	.9	467	
	30-45	58	.7	121	.6	429	
	45-75	59	.7	120	.6	433	
	75-120	64	.8	123	.6	428	

* Gouty subjects.

when administered orally in 1-3 g dosage.

5. *Side reactions.* Like nicotinic acid, which has a similar structure, pyrazinoic acid and pyrazinamide may elicit transitory peripheral vasodilatation and dizziness, particularly in 3 g dosage. This reaction was marked in Case S.Z. and may account in part for the fall in C_{inulin} and C_{PAH}. No other side reactions were noted. Of interest is the failure to precipitate acute gouty arthritis in any of our gouty subjects. This has been noted by others, but only after several weeks of therapy, and presumably only in patients with gouty diathesis.

Discussion. It has been pointed out previously(7) that a variety of uricosuric drugs, when given in low dosage, may cause a transitory fall in C_{urate}; occasionally to as low as 50% of the pre-medication level in the case of salicylate. The reduction in C_{urate} was much more pronounced with pyrazinamide and pyrazinoic acid, however, consistently reaching levels of 5-10% of the control value when appropriate dosages were employed. No uricosuric effect was noted under the conditions of these experiments.

This striking reduction in C_{urate} cannot be ascribed to a decrease in filtered urate. It occurred when C_{inulin} was not affected, and ultrafiltration of the serum urate in 2 cases, before and again 4 and 8 hours after medication when the serum urate was distinctly elevated, showed no change in the virtually complete filtrability of the serum urate. It would seem to follow that the reduction in C_{urate} was due to enhanced tubular reabsorption of urate, calculated to exceed 99% of the filtered load in most cases. Another possibility, previously considered(7), is that tubular secretion of urate may occur in man, and that the drugs in question inhibit this process. In this event, the marked (in large dosage almost complete) suppression of UV_{urate} implies that the filtered load of urate presented to the tubules is virtually completely reabsorbed, and that the urate appearing in the urine derives largely from tubular secretion of urate. This process, then, would be analogous to that postulated for potassium.

In this connection, the relative independence of tubular secretory mechanisms for PAH might be pointed out. Previous studies with probenecid(4) and phenylbutazone(8), in doses which markedly increase C_{urate} by inhibition of tubular reabsorption of urate, showed more marked lowering of Tm_{PAH} in equivalent dosage. Beyer(9) has commented in some detail on this aspect of the problem of tubular transport mechanisms.

The data indicate that pyrazinoic acid, a product of hydrolysis of pyrazinamide in the body, produces more rapid and more marked reduction in C_{urate} than does pyrazinamide in equivalent dosage. This suggests that the free acid may be largely or wholly responsible for the effect on urate transport.

Summary. 1. The effect of pyrazinamide and pyrazinoic acid on the renal clearance of urate, inulin and p-aminohippurate, in some instances also Tm_{PAH}, was studied in 10 human subjects. 2. A rapid and marked reduction in urate clearance was noted, without decrease in the filtered urate load. The implications as to the renal mechanisms regulating urate excretion are discussed. 3. Data indicating that the effect of pyrazinamide is ascribable largely to the action of pyrazinoic acid are presented.

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Antigenicity of Chondroitin Sulfate.* (23451)

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The recent reports of Glynn and Holborow (1) in which they observed the production of complete antigens from vegetable polysaccharide haptens (agar) and the conversion of tissue polysaccharides (chrondroitin sulfate) to haptens(2) by hemolytic streptococci and other bacteria are of great interest for 2 reasons. First, because the lesions of rheumatic fever occur in connective tissue and it has been postulated that these lesions might be due to an auto-antigen, auto-antibody reaction occurring in connective tissue(3) and second, the hemolytic streptococcus is intimately involved in the pathogenesis of rheumatic fever. The findings of Glynn and Holborow would appear to be of great potential significance and every effort should be made to confirm them.

According to Meyer(4) 6 separate mucopolysaccharides have been isolated from the ground substance of connective tissue, hyaluronic acid, chondroitin sulfate A, B and C, chondroitin and keratosulfate. Chondroitin sulfate A is the main component of hyaline cartilage but it contains some chondroitin sulfate C which has also been found in heart valves, tendon, aorta and umbilical cord. None of these components has been shown to be antigenic alone. It was the purpose of this experiment to attempt to render chondroitin sulfate antigenic.

Materials and methods. Chondroitin sulfate was prepared from fresh wet human costal cartilage stored at 0°C for 2-3 months according to the method of Einbinder and Schubert (5). It was assayed by the Dische(6) glucuronic acid method with glucuronolactone as the standard and was found to be 65% pure chondroitin sulfate. Bovine chondroitin sulfate† of 70% purity was tested along with the samples. Readings were taken in a Coleman spectrophotometer at 540 mu. Strains of group A type 3 hemolytic streptococcus or hemolytic *Staphylococcus aureus* were grown for 18 hours at 37°C in beef heart infusion broth. The bacterial cells were removed by centrifugation, suspended in a 1% solution of chondroitin sulfate in 0.85% saline, incubated at 37°C for 2 hours and then heat-killed at 60°C for 75 minutes. The bacterial cells then were washed 3 times in normal saline and finally resuspended in saline. This preparation of washed, heat-killed bacterial cells *without* chondroitin sulfate, bacterial cells incubated *with* chondroitin sulfate and a 1% solution of chondroitin sulfate were the materials injected into young adult albino or chinchilla rabbits weighing from 3-5 lbs according to the dose, route and schedule outlined in Table I. The rabbits were bled at the times shown in Table I. The serum containing merthiolate at a strength of 1:10,000 was stored at 4-8°C. Tests for precipitating antibodies against chondroitin sulfate were performed using capillary tubes. An optimal proportion method was used overlaying serial dilutions of 0.1% chondroitin sulfate in normal saline with serial dilutions of each serum specimen. Each of the undiluted sera and a 1:4, 1:16, 1:32 and 1:64 dilution of each serum was tested with each of an undiluted sample and a 1:4, 1:16, 1:32 and 1:64 dilution of chondroitin sulfate. The tubes, incubated for 2 hours at 37°C, then placed in the refrigerator at 4-8°C overnight, were observed for precipitate formation both before and after incubation at 37°C and 4-8°C. Following completion of the "immunizing" series of injections, each rabbit was skin tested. One-tenth ml of 1% chondroitin sulfate was injected intradermally and observed for evidence of reaction immediately, 6 hours and 24 hours after injection.

Results. There was no evidence of the formation of precipitating antibodies against chondroitin sulfate in any of the rabbits' sera. Slight erythema, 4 mm in diameter, was seen

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* Aided by grant from S. E. Massengill Co.

† General Biochemicals Inc.

TABLE I. Dose, Route, and Date of Administration of Each Antigen to Each Rabbit.

Rabbit #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Antigen	Chondroitin sulfate + streptococci					Chondroitin sulfate		Strepto-cocci		Chondroitin sulfate + staphylococci				Staphylo-cocci	
Day of exp.	I.V.	I.V.	I.V.	I.M.	I.M.	I.V.	I.V.	I.V.	I.V.	I.V.	I.V.	I.M.	I.M.	I.V.	I.V.
	Dose of antigen in ml														
1*	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1
3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3
5	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6
8*	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8
10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
12	”	”	”	”	”	”	”	”	”	”	”	”	”	”	”
15*	”	”	”	”	”	”	”	”	”	”	”	”	”	”	”
17	”	”	”	”	”	”	”	”	”	”	”	”	”	”	”
19*	”	”	”	”	”	”	”	”	”	”	”	”	”	”	”

* Blood specimen drawn.
I.V. = intrav.; I.M. = intramusc.

about the injection site of one rabbit which received chondroitin sulfate plus streptococci and 6-8 mm erythema in a rabbit which received streptococci alone. There was no evidence of skin reactivity in any of the other rabbits.

Comments. The results of these experiments, in which there was no evidence of antigenicity of chondroitin sulfate either in the role of a hapten or as a direct antigen as measured by precipitating antibodies and skin sensitivity, are at variance with those of Glynn and Holborow(2). However, these authors have since indicated that they have been unable to repeat their original work and they suspect the precipitating antibodies observed by them were "in fact directed against blood group like substances and not against chondroitin sulphate itself."‡ It would appear at the present time that chondroitin sulfate by itself or in combination with the group A streptococcus or *Staphylococcus aureus* has not been shown to be antigenic nor to behave as a hapten. These results are similar to those of previous experiments in which hyaluronic acid proved to be non-antigenic(7).

Summary. Chondroitin sulfate extracted from human costal cartilage was prepared in 3 different ways for inoculation into rabbits, (1) a 1% solution, (2) a 1% solution incubated with heat-killed, group A, type 3, hemolytic streptococcus, and (3) a 1% solution incubated with heat-killed *Staphylococcus aureus.* These 3 preparations were injected into rabbits in an attempt to elicit antibody production. There was no evidence of the formation of precipitating antibodies against chondroitin sulfate in any of the rabbits' sera nor was there any clear indication of the acquisition of skin sensitivity by any of the rabbits.

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Inhibition of Antibacterial Activity of Cycloserine by Alpha-Alanine.[*]

(23452)

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The antibacterial properties of cycloserine have been described by various groups of investigators(1-3). This compound, identified as D-4-amino-3-isoxazolidone(4), has its greatest activity against Gram-positive cocci and the tubercle bacillus. Evidence has been presented(2) which indicates that cycloserine may be more active *in vivo* than *in vitro*. This observation suggested the possibility that certain ingredients of culture media used for testing its activity might be inhibitory(5). Consequently, a study was undertaken to determine the potential interference of the antibacterial properties of this uniquely simple compound by various growth stimulating compounds. This study revealed that the amino acid, alpha-alanine, specifically inhibited the antibacterial action of cycloserine on staphylococci.

Methods. Various strains of staphylococcus were selected as test organisms. Previous studies(6) have shown that members of this group of organisms may be cultivated in fluid defined media containing salts, vitamins, and 8 to 10 amino acids. The minimal inhibitory concentration (MIC) of cycloserine[†] against various strains of staphylococci in such media was determined. Simultaneous determinations of the MIC were also made in the same defined media to which had been added, separately, varying quantities of vitamins, amino acids, purines, and pyrimidines not essential for growth. The potential effect on cycloserine of growth factors essential for growth was determined by testing the activity of the antibiotic in media in which the concentration of each essential factor was increased 10 to 100 fold over that required for growth. Growth measurements were made after incubation at 37°C for 48 hours. These were made either on the Evelyn photoelectric colorimeter, or by gross determination of the presence or absence of growth. End points of 90 to 100% inhibition of growth were used in determining the MIC of the antibiotic. All strains of staphylococci studied had been isolated from clinical infections. These were transferred monthly on trypticase soy agar slants and stored in the refrigerator between experiments.

Results. An exhaustive study of most of the more common growth-promoting compounds revealed that alpha-alanine was the only compound which specifically inhibited activity of cycloserine on staphylococci. In Table I are shown the concentrations of cycloserine required for inhibition of growth of 3 strains of staphylococci grown in fluid defined medium containing varying amounts of alanine. Except for minor differences in susceptibility, the results with all 3 strains are quite similar. With each strain, the ratio of the concentration of cycloserine required for inhibition of growth to that of alanine over a wide range

TABLE I. Concentration of Cycloserine Required for Inhibition of Staphylococci in the Presence of Alanine.

Staph.	Alanine, µg/ml	Cycloserine,* µg/ml	Inhibition index†
7739R	0	.32	25
	.06	1.60	
	.32	8.0	
	1.6	40.0	
	8.0	200.0	
	40.0	1000	
	200	1000	
2255	0	1.0	20
	.5	10	
	5.0	100	
	50	1000	
Van	0	.2	10
	.1	1.0	
	1.0	10	
	10	100	
	100	1000	

* Minimal conc. producing 90 to 100% inhibition of growth.

† Ratio of $\frac{\text{Cycloserine conc.}}{\text{Alanine conc.}}$.

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[*] This investigation was supported by a reasearch grant from the Nat. Inst. of Health, P.H.S.

[†] Supplied by Eli Lilly & Co., Indianapolis, Ind. and the Pfizer Laboratories, Brooklyn, N. Y.

TABLE II. Inhibition of Growth of Various Organisms by Cycloserine in Presence and Absence of Alanine.

Culture	Alanine, μg/ml	Cycloserine, μg/ml 0	.32	1.6	8	40	200
		% inhibition of growth					
Staph. 2255	0	0	50	100	100	100	100
	50	0	0	0	0	0	0
E. coli	0	0	0	0	0	50	100
	50	0	0	0	0	0	100
S. flexneri	0	0	0	0	0	100	100
	50	0	0	0	0	0	100
B. cereus	0	0	0	0	0	0	100
	50	0	0	0	0	0	100

of concentrations of both compounds is remarkably constant. Only in the presence of large quantities of cycloserine, 1000 μg/ml or greater, does this appear not to be true.

The ability of alpha-alanine to interfere with activity of cycloserine does not appear to be true in the case of certain other species of organisms tested. By a similar procedure, the activity of cycloserine on single strains of *E. coli, Shigella flexneri and B. cereus* was determined in defined media in the presence and absence of alpha-alanine, 50 μg/ml. A culture of staphylococcus was likewise tested for comparative purposes. In Table II are shown the results of this experiment. The strain of staph. tested was considerably more susceptible than the other species of organisms. Furthermore, alanine in a concentration, 50 μg/ml, which definitely reduced the activity of cycloserine on the strain of staph., had a negligible effect on the activity of the antibiotic against the latter organisms.

TABLE III. Comparison of Inhibition of Cycloserine by Alanine and That by Related Compounds.

Compound	Inhibition of cycloserine,* % of alanine activity†
DL-alpha-alanine	100
L-alpha-alanine	100
D-alpha-alanine	75-90
Phenylalanine	0
Beta-alanine	0
Sodium pyruvate	0
Sarcosine	0
Alanylglycine‡	50
Glycylalanine‡	50
Serine	0
Alpha aminobutyric acid	1.0
Beta " "	0

* Staph. 7739 used as test strain.

† Comparisons made on wt basis.

‡ Other peptides active to extent of alpha-alanine content by wt.

The observation that alanine competitively antagonizes the activity of cycloserine on staphylococci indicated the need for studies to determine whether other compounds related to or structurally similar to alpha-alanine and/or cycloserine had similar antagonistic properties. In Table III are shown the results of such experiments comparing the activity of alpha-alanine with several such compounds. Both the D and L isomers of alpha-alanine antagonized the activity of the antibiotic. Aside from the peptides which were active to the extent of their alanine content, the only other compound with alanine-like activity on cycloserine was alpha-aminobutyric acid which had 1% the activity. It is evident that inhibition of cycloserine is a relatively specific function of alpha-alanine. Any major deviation from its normal structure results in complete loss of activity. Quite surprising was the observation that the amino acid, DL-serine, a degradation product of cycloserine, had no effect upon the antibiotic.

TABLE IV. Effect of Alanine on Antistaphylococcal Activity of Various Antibiotic Agents.

Agent	MIC of agent, μg/ml* In basal medium	In basal medium + alanine, 50 μg/ml
Cycloserine	.2	100
Penicillin	.04	.08
Magnamycin	.4	.2
Erythromycin	.1	.1
Tetracycline	3.1	6.2
Chloromycetin	6.2	6.2
Neomycin	.45	.45
Streptomycin	.8	.8
Oleandomycin	3.1	3.1
Novobiocin	3.1	1.6
Vancomycin	.4	.8

* Penicillin, units/ml.

In an attempt to determine whether the action of alanine was specific only for cycloserine, similar experiments were carried out with several of the other antibiotic agents. As shown in Table IV, alanine failed to affect the activity of any of the other agents. Under the same experimental conditions, however, the concentration of cycloserine required for inhibition of a strain of staphylococcus was 500 times greater in the presence of alpha-alanine, 50 μg/ml, than in its absence.

Discussion. Several features of the phenomenon described in this report are quite significant. The ability of alpha-alanine to antagonize the action of cycloserine on staphylococci and not that on other organisms tested is not surprising. This may be attributed to the fact that the nutritional requirements of staphylococci are more complex. Of considerable significance is the fact that the alanine molecule must be unaltered for it to antagonize the antibiotic. Minor changes in the structure of the amino acid results in its failure to antagonize. Of particular significance in this respect is the complete loss of activity resulting from the changing of the amino group to the beta-position. In a similar fashion, any substitution on the methyl group results in complete loss of activity. As yet, we have been unsuccessful in locating a compound capable of relieving the inhibition of cycloserine by alanine.

The constancy of the ratio of the two compounds over a fairly broad range of concentrations is characteristic of that of a competitive type of inhibition(7). This lends support to the belief that cycloserine acts on staphylococci by virtue of its ability to interfere in some way with the metabolism of alanine. No doubt the greater susceptibility of staphylococci to the antibiotic than that of the other species studied is related to the specific interference in the metabolism of alanine by this group of organisms.

Summary. Alpha-alanine inhibits antibacterial activity of cycloserine on staphylococci. The inhibition appears to be of a competitive nature. This amino acid does not inhibit activity of the antibiotic on other organisms studied. Alpha-alanine does not interfere with the action of other antibiotic agents.

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